

Appl. No. : 10/063,587
Filed : May 3, 2002

REMARKS

Claims 1-5 remain pending in the instant application. No amendments have been made. Applicants respond below to the specific rejections set forth in the Office Action mailed December 13, 2004.

Rejection under 35 U.S.C. §101 – Utility

The PTO has maintained the rejection of Claims 1-5 under 35 U.S.C. § 101 as lacking patentable utility. The PTO argues that the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility for the reasons of record on pages 2 and 3 of the previous Office Action.

Specifically, according to the PTO, Applicants' previous arguments regarding utility were not persuasive. The PTO argues that utility requires that the skilled artisan be able to use the claimed invention, and that the specification does not provide a specific and substantial or a well-established use. The PTO further argues that Applicants have provided only a single analysis without any relative range for basing a utility of underexpression. The PTO asserts that it "is not disclosed what type(s) of lung or stomach tumor was analyzed," and that it "is not clear if the findings can be generalized to all tumors from that tissue type." Also, the PTO argues that the skilled artisan would not know if the results were significant or under what conditions the difference in expression could be detected.

The PTO makes various other arguments on pages 3 and 4 of the Final Office Action, including:

[W]ithout knowing the range of variation there is insufficient guidance. If a clinician took a stomach tissue sample from a patient with a suspected stomach cancer, what is the likelihood that when compared with normal tissue, the level of nucleic acid of SEQ ID NO:77 from the patient would be lower? How many samples would be needed? What sensitivity would be needed? Would the normal tissue have to be a pooled sample or could it be from a single individual?

The PTO further argues that "[t]he statement that the relative difference in expression is what is important is generally true, but without more specifics about necessary sample size, expression level range for normal and tumor tissues, types of stomach or lung tissue that can be used, and other questions, the specification has not provided the invention in a form readily usable by the skilled [artisan] such that significant further experimentation was unnecessary."

Appl. No. : 10/063,587
Filed : May 3, 2002

With regard to the correlation between nucleic acid expression and expression of the corresponding protein, the PTO also continues to argue that there is no evidence that the polypeptide of PRO1357 is underexpressed in stomach tumors or lung tumors. In support the PTO argues that the data are insufficient because only relative expression data was presented. The PTO argues that there is no evidentiary support to the assertions by Dr. Polakis that it remains the central dogma in molecular biology that increased mRNA levels are predictive of corresponding increased levels of the encoded polypeptide.

The PTO references Hu et al. (2003, Journal of Proteome Research 2:405-412) as refuting Applicants assertions regarding the correlation between nucleic acid expression and polypeptide expression. Also, the PTO attempts to refute the other Declarations and references submitted by Applicants by referencing papers by Haynes et al. (1998, Electrophoresis 19:1862-1871) and Konopka et al. (1986, PNAS 83:4049-4052).

Applicants reiterate that the claimed invention has utility in diagnosing cancer, specifically in the diagnosis of stomach cancer and lung cancer. The specification in Example 18 discloses data showing that the nucleic acid of SEQ ID NO:77 is more highly expressed in normal stomach tissue or normal lung tissue compared to stomach tumor or lung tumor, respectively. The data in Example 18 is more than sufficient to satisfy the correct utility standard under 35 U.S.C. § 101. Nonetheless, Applicants have provided various declarations by those of skill in the art, literature references, and textbook passages further supporting the claimed utility. The references cited by the PTO to refute the claimed utility do not actually support a lack of utility. In view of this and the discussion below, Applicants respectfully submit that the claimed antibodies have a credible, substantial, and specific utility.

Utility need NOT be proven to an Absolute Certainty – a Correlation between the Evidence and the Asserted Utility is Sufficient

Compliance with 35 U.S.C. § 101 is a question of fact. *Raytheon v. Roper*, 724 F.2d 951, 956, 220 USPQ 592, 596 (Fed. Cir. 1983) cert. denied, 469 US 835 (1984). The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the evidence, or “more likely than not” standard. *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). This is stated explicitly in the M.P.E.P.:

Appl. No. : 10/063,587
Filed : May 3, 2002

[T]he applicant does not have to provide evidence sufficient to establish that an asserted utility is true “beyond a reasonable doubt.” Nor must the applicant provide evidence such that it establishes an asserted utility as a matter of statistical certainty. Instead, evidence will be sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not. M.P.E.P. at § 2107.02, part VII (2004) (emphasis in original, internal citations omitted).

The PTO has the initial burden to offer evidence “that one of ordinary skill in the art would reasonably doubt the asserted utility.” *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). Only then does the burden shift to the Applicant to provide rebuttal evidence. *Id.* As stated in the M.P.E.P., such rebuttal evidence does not need to absolutely prove that the asserted utility is real. Rather, the evidence only needs to be reasonably indicative of the asserted utility.

In *Fujikawa v. Wattanasin*, 93 F.3d 1559, 39 U.S.P.Q.2d 1895 (Fed. Cir. 1996), the Court of Appeals for the Federal Circuit upheld a PTO decision that *in vitro* testing of a novel pharmaceutical compound was sufficient to establish practical utility, stating the following rule:

[T]esting is often required to establish practical utility. But the test results **need not absolutely prove** that the compound is pharmacologically active. All that is required is that the tests be “*reasonably* indicative of the desired [pharmacological] response.” In other words, there must be **a sufficient correlation** between the tests and an asserted pharmacological activity so as to convince those skilled in the art, **to a reasonable probability**, that the novel compound will exhibit the asserted pharmacological behavior.” *Fujikawa v. Wattanasin*, 93 F.3d 1559, 1564, 39 U.S.P.Q.2d 1895 (Fed. Cir. 1996) (internal citations omitted, bold emphasis added, italics in original).

While the *Fujikawa* case was in the context of utility for pharmaceutical compounds, the principles stated by the Court are applicable in the instant case where the asserted utility is for a diagnostic use – utility does not have to be established to an absolute certainty, rather, the evidence must convince a person of skill in the art “to a reasonable probability.” In addition, the evidence need not be direct, so long as there is a “sufficient correlation” between the tests performed and the asserted utility.

Here, as discussed more fully below, the evidence of record, including the declarations by those of skill in the art and the teachings of the specification, is convincing to a person of skill in the art “to a reasonable probability.”

Appl. No. : 10/063,587
Filed : May 3, 2002

The Court in *Fujikawa* relied in part on its decision in *Cross v. Iizuka*, 753 F.2d 1040, 224 U.S.P.Q. 739 (Fed. Cir. 1985). In *Cross*, the Appellant argued that basic *in vitro* tests conducted in cellular fractions did not establish a practical utility for the claimed compounds. Appellant argued that more sophisticated *in vitro* tests using intact cells, or *in vivo* tests, were necessary to establish a practical utility. The Court in *Cross* rejected this argument, instead favoring the argument of the Appellee:

[I]n *vitro* results...are generally predictive of *in vivo* test results, i.e., there is a **reasonable correlation** therebetween. Were this not so, the testing procedures of the pharmaceutical industry would not be as they are. [Appellee] has not urged, and rightly so, that there is an invariable exact correlation between *in vitro* test results and *in vivo* test results. Rather, [Appellee's] position is that successful *in vitro* testing for a particular pharmacological activity establishes a **significant probability** that *in vivo* testing for this particular pharmacological activity will be successful. *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 U.S.P.Q. 739 (Fed. Cir. 1985) (emphasis added).

The *Cross* case is very similar to the present case. Here, no additional sophisticated testing or further research is required to establish the utility of the nucleic acids, the encoded polypeptides or the claimed antibodies in cancer diagnostics. As with *in vitro* testing in the pharmaceutical industry, those of ordinary skill in the art recognize to a reasonable probability that a showing of differential expression of mRNA in cancerous cells compared to normal cells indicates a real world utility in cancer diagnostics for the nucleic acids, their encoded polypeptides and the antibodies to the polypeptides. One of ordinary skill in the art would rely upon the differential expression data in Example 18 as reasonably indicating a real world use for the nucleic acids, the encoded polypeptides and the claimed antibodies. Those of ordinary skill in the art recognize a reasonable correlation between differential expression in cancerous versus non-cancerous cells and utility in distinguishing between those cells.

Also, as in *Cross*, Applicants here do not argue that there is “an invariable exact correlation” between differential expression and diagnostic markers. Instead, Applicants’ position detailed below is that the data in Example 18 are reliable and significant, as well as more than sufficient to establish a “significant probability” that the differential expression of the nucleic acids and PRO1357 polypeptides in cancerous versus non-cancerous tissue provides diagnostic utility for the same, as well as antibodies to the same, based on “a reasonable correlation therebetween.” In order to satisfy the proper utility standard, no further research or

Appl. No. : 10/063,587
Filed : May 3, 2002

testing is required. One of skill in the art more likely than not would recognize utility for the claimed antibodies based upon the differential expression data in Example 18.

Also, those of skill in the field of biotechnology rely on the reasonable correlation that exists between gene expression and protein expression (see below). Were there no reasonable correlation between the two, the techniques that measure gene levels such as microarray analysis, differential display, and quantitative PCR would not be so widely used by those in the art. As in *Cross*, Applicants here do not argue that there is “an invariable exact correlation” between gene expression and protein expression. Instead, Applicants’ position detailed below is that a measured change in gene expression in cancer cells establishes a “significant probability” that the expression of the encoded polypeptide in cancer will also be changed based on “a reasonable correlation therebetween.”

Even assuming that the PTO has met its initial burden to offer evidence that one of ordinary skill in the art would reasonably doubt the truth of the asserted utility, Applicants assert that they have met their burden of providing rebuttal evidence such that it is more likely than not those skilled in the art, to a reasonable probability, would believe that the PRO1357 polypeptide and antibodies thereto are useful as diagnostic tools for stomach and lung cancer. Applicants further address and rebut the specific points raised by the Examiner in the Final Office Action.

The Differential Expression of the PRO1357 mRNA confers Utility upon the Nucleic Acids, the PRO1357 Polypeptide, and the Claimed Antibodies

Applicants submit that the evidence of record demonstrates a substantial and specific utility for the claimed antibodies. No additional testing is required to establish to “a reasonable probability” the utility of the claimed antibodies in cancer diagnostics. Those of skill in the art recognize a “real world” utility based upon the data set forth in the application as filed. As set forth in Example 18, nucleic acids encoding the PRO1357 polypeptide are more highly expressed in normal stomach tissue or normal lung tissue compared to stomach tumor or lung tumor, respectively. Thus, one of skill in the art would recognize that it is more likely than not that the polypeptide is more highly expressed in normal stomach tissue or normal lung tissue compared to stomach tumor or lung tumor, respectively. It is this differential expression of the nucleic acids and the polypeptides in the respective cancer and non-cancerous cells that makes the nucleic acids, the polypeptides and the antibodies to the polypeptides useful in the diagnosis of

Appl. No. : 10/063,587
Filed : May 3, 2002

cancer, as it serves as the basis for detecting a difference between cancerous and non-cancerous cells.

Nonetheless, the PTO continues to argue that the data in Example 18 are insufficient and do not provide an immediate use for the claimed subject matter. Applicants strongly disagree. The MPEP states:

Office personnel must be careful not to interpret the phrase ‘immediate benefit to the public’ or similar formulations in other cases to mean that products or services based on the claimed invention must be ‘currently available’ to the public in order to satisfy the utility requirement. See, e.g., *Brenner v. Manson*, 383 U.S. 519, 534-35, 148 USPQ 689, 695 (1966). Rather, any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a ‘substantial’ utility. Courts have repeatedly found that the mere identification of a pharmacological activity of a compound that is relevant to an asserted pharmacological use provides an ‘immediate benefit to the public’ and thus satisfies the utility requirement. See *Nelson v. Bowler* 626 F.2d 853, 856, 206 USPQ 881, 883 (CCPA 1980).

M.P.E.P. at 2107.01 (Emphasis in original).

The data in Example 18 are sufficient to provide an “immediate benefit to the public” or a “real world” use. Applicants have identified molecules that can be used as cancer markers. This knowledge provides an immediate public benefit. Under § 101 the utility does not have to be an FDA approved use, one that is ready for commercial sale, or even one that is ready for clinical use. Use as a cancer marker based upon the differential expression data in Example 18 are sufficient for utility under § 101. The data show that the nucleic acids encoding the PRO1357 polypeptide are differentially expressed in stomach and lung tissue compared to cancerous stomach and lung tissue, respectively. Example 18 explains that standard techniques and controls were utilized. Specifically, the widely accepted technique of PCR was used to determine “whether the polynucleotides tested were more highly expressed, less expressed, or whether expression remained the same in tumor tissue as compared to its normal counterpart.” Furthermore, the first Grimaldi Declaration explains the techniques and protocols, stating that the gene expression studies reported in Example 18 of the instant application were performed using pooled samples of normal and of tumor tissues. With regard to reliability, Mr. Grimaldi explains that:

The DNA libraries used in the gene expression studies were made from pooled samples of normal and of tumor tissues. Data from pooled samples is more likely to be accurate than data obtained from a sample from a single individual. That is,

Appl. No. : 10/063,587
Filed : May 3, 2002

the detection of variations in gene expression is likely to represent a more generally relevant condition when pooled samples from normal tissues are compared with pooled samples from tumors in the same tissue type. (Paragraph 5).

Furthermore, the Declaration of Grimaldi explained the significance of the results and that the methodology utilized in Example 18 indicates that the difference in expression levels between normal and cancerous cells is at least two fold. In paragraphs 6 and 7, Mr. Grimaldi explains that the semi-quantitative analysis employed to generate the data of Example 18 is sufficient to determine if a gene is over- or underexpressed in tumor cells compared to corresponding normal tissue. "Because this technique relies on the visual detection of ethidium bromide staining of PCR products on agarose gels, it is reasonable to assume that any detectable differences seen between two samples will represent at least a two fold difference in cDNA." He also states that the results of the gene expression studies indicate that the genes of interest "can be used to differentiate tumor from normal." He explains that "[t]he precise levels of gene expression are irrelevant; what matters is that there is a relative difference in expression between normal tissue and tumor tissue." (Paragraph 7). Thus, since it is the relative level of expression between normal tissue and suspected cancerous tissue that is important, the precise level of expression in normal tissue is irrelevant. Likewise, there is no need for additional quantitative data to compare the level of expression in normal and tumor tissue. As Mr. Grimaldi states, **"If a difference is detected, this indicates that the gene and its corresponding polypeptide and antibodies against the polypeptide are useful for diagnostic purposes, to screen samples to differentiate between normal and tumor (emphasis added)."** Thus, there is guidance as to the levels of expression, particularly, that at a minimum, there is at least a two fold difference between the expression levels in cancerous cells compared to the corresponding non-cancerous cells. Therefore, contrary to the conclusions in the Final Office Action, Applicants have established that one of skill in the relevant art would recognize that there is a real world significance to the differential expression data set forth in the specification, and that a reasonable correlation exists between the data and the claimed antibodies in diagnostics.

Despite the presented data, the various declarations and the submitted literature references in support, many of the arguments made by the PTO attempt to refute the operability of the claimed invention.

Applicants remind the PTO that:

Appl. No. : 10/063,587
Filed : May 3, 2002

A small degree of utility is sufficient . . . The claimed invention must only be capable of performing some beneficial function . . . An invention does not lack utility merely because the particular embodiment disclosed in the patent lacks perfection or performs crudely . . . A commercially successful product is not required . . . Nor is it essential that the invention accomplish all its intended functions . . . or operate under all conditions . . . partial success being sufficient to demonstrate patentable utility . . . In short, the defense of non-utility cannot be sustained without proof of total incapacity. See *E.I. du Pont De Nemours and Co. v. Berkley and Co.*, 620 F.2d 1247, 1260 n.17, 205 USPQ 1, 10 n.17 (8th Cir. 1980). If an invention is only partially successful in achieving a useful result, a rejection of the claimed invention as a whole based on a lack of utility is not appropriate. See *In re Brana*, 51 F.3d 1560, 34 USPQ2d 1436 (Fed. Cir. 1995); *In re Gardner*, 475 F.2d 1389, 177 USPQ 396 (CCPA), *reh'g denied*, 480 F.2d 879 (CCPA 1973); *In re Marzocchi*, 439 F.2d 220, 169 USPQ 367 (CCPA 1971).

M.P.E.P. at 2107.01 (emphasis in original).

Here, the claimed subject matter can be used by those of skill in the art to differentiate cancerous versus non-cancerous stomach and lung cells. Thus, the claimed subject matter is operative and the various questions raised by the PTO do not defeat the utility under § 101.

For these reasons, Applicants submit that one of ordinary skill in the art would reasonably recognize the utility of the claimed antibodies.

Applicants have Established that the Accepted Understanding in the Art is that there is a Direct Correlation between Comparative mRNA Levels and the Level of Expression of the Encoded Protein in Normal versus Cancerous Tissue

Applicants maintain that it is more likely than not that the encoded polypeptide is more highly expressed in normal stomach tissue or normal lung tissue compared to stomach tumor or lung tumor tissue respectively. Applicants reiterate that the evidence of record establishes a correlation between mRNA expression and protein expression. As stated above, the standard for utility is not absolute certainty, but rather whether one of skill in the art would be more likely than not to believe the asserted utility. The working hypothesis among those skilled in the art is that there is a direct correlation between mRNA levels and protein levels. Despite some teachings in the art of certain genes that do not fit within this paradigm, which are exceptions rather than the rule, in the vast majority of cases, the combined teachings in the art, exemplified by the previously submitted papers, for example by Orntoft et al., Hyman et al. and Pollack et al. and the previously submitted Grimaldi and Polakis declarations, overwhelmingly teach that gene

Appl. No. : 10/063,587
Filed : May 3, 2002

expression influences mRNA expression and protein levels. Thus, one of skill in the art would reasonably expect, in this instance, based on the gene expression data for the PRO1357 gene, that the PRO1357 protein is concomitantly over-expressed in normal stomach tissue compared to stomach tumor tissue, and in normal lung tissue compared to lung tumor tissue.

The statements of Grimaldi and Polakis are further supported by the teachings of two leading textbooks. Molecular Biology of the Cell is a leading cell biology textbook in the field (Bruce Alberts, *et al.*, Molecular Biology of the Cell (4th ed. 2002), submitted herewith as Exhibit 1). Figure 6-3 on page 302 illustrates the basic principle that there is a correlation between increased gene expression and increased protein expression. The accompanying text states that “a cell can change (or regulate) the expression of each of its genes according to the needs of the moment – *most obviously by controlling the production of its mRNA.*” Molecular Biology of the Cell at 302, emphasis added. Similarly, figure 6-90 on page 364 illustrates the path from gene to protein. The accompanying text states that while potentially each step can be regulated by the cell, “the initiation of transcription is the most common point for a cell to regulate the expression of each of its genes.” Molecular Biology of the Cell at 364. This point is repeated on page 379, where the authors state that of all the possible points for regulating protein expression, “[f]or most genes transcriptional controls are paramount.” Molecular Biology of the Cell at 379.

Also, support for Applicants’ position can be found in the Lewin textbook (Genes VI (1997) CH 29, pp. 847-848; submitted herewith as Exhibit 2) which also states that “having acknowledged that control of gene expression can occur at multiple stages, and that production of RNA cannot inevitably be equated with production of protein, it is clear that the overwhelming majority of regulatory events occur at the initiation of transcription” (emphasis added).

Still more support is found in Zhigang *et al.*, World Journal of Surgical Oncology 2:13, 2004, submitted herewith as Exhibit 3. Zhigang studied the expression of prostate stem cell antigen (PSCA) protein and mRNA to validate it as a potential molecular target for diagnosis and treatment of human prostate cancer. The data showed “a high degree of correlation between PSCA protein and mRNA expression” (see page 4 of Exhibit 3). Of the samples tested, 81 out of 87 showed a high degree of correlation between mRNA expression and protein expression. The authors conclude that “it is demonstrated that PSCA protein and mRNA overexpressed in human prostate cancer, and that the increased protein level of PSCA was resulted from the upregulated transcription of its mRNA.” Exhibit 3, page 6. Even though the correlation between mRNA

Appl. No. : 10/063,587
Filed : May 3, 2002

expression and protein expression occurred in 93% of the samples tested, not 100%, the authors state that "PSCA may be a promising molecular marker for the clinical prognosis of human Pca and a valuable target for diagnosis and therapy of this tumor." Exhibit 3, page 7.

Further, Meric *et al.*, Molecular Cancer Therapeutics, vol. 1, 971-979 (2002), submitted herewith as Exhibit 4, states the following:

The **fundamental principle** of molecular therapeutics in cancer is to exploit the differences in gene expression between cancer cells and normal cells...[M]ost efforts have concentrated on identifying differences in gene expression at the level of mRNA, which can be attributable to either DNA amplification or to differences in transcription. Meric *et al.* at 971 (emphasis added).

This statement provides additional support for Applicants' asserted utility. It is true that there is no *necessary* correlation between gene expression and protein expression because there are other mechanisms for regulating gene expression. However, were there no significant correlation between gene expression and protein levels, exploiting differences in gene expression between cancer cells and normal cells would not be a "fundamental principle of molecular therapeutics in cancer." Moreover, as mentioned above, Applicants need not establish a *necessary* connection between gene expression and protein expression. Rather, there need only be a *reasonable* correlation between the evidence offered and the asserted utility such that it is more likely than not that a person of skill in the art would be convinced, to a reasonable probability, that the asserted utility is true.

The PTO relies on Hu *et al.* (J. Proteome Res., 2(4):405-12 (2003)) to support its assertion that the literature cautions researchers from drawing conclusions based on small changes in transcript expression levels between normal and cancerous tissue. Applicants respectfully submit that Hu does not satisfy the PTO's burden to offer evidence that one of ordinary skill in the art would reasonably doubt the truth of the asserted utility.

In Hu, the researchers used an automated literature-mining tool to summarize and estimate the relative strengths of all human gene-disease relationships published on Medline. They then generated a microarray expression dataset comparing breast cancer and normal breast tissue. Using their data-mining tool, they looked for a correlation between the strength of the literature association between the gene and breast cancer, and the magnitude of the difference in expression level. They report that for genes displaying a 5-fold change or less in tumors compared to normal, there was no evidence of a correlation between altered gene expression and

Appl. No. : 10/063,587
Filed : May 3, 2002

a *known* role in the disease. *See* Hu at 411. However, among genes with a 10-fold or more change in expression level, there was a strong correlation between expression level and a *published* role in the disease. *See id.* at 412. Importantly, Hu reports that the observed correlation was only found among estrogen receptor-positive tumors, not ER-negative tumors. *See id.*

The general findings of Hu are not surprising – one would expect that genes that have the greatest change in expression in a disease would be the first targets of research, and therefore have the strongest *known* relationship to the disease as measured by the number of reports of a connection in the literature. But this does not mean that genes, and their corresponding proteins, with a lower level of change in expression are not important or cannot be used as molecular markers of the disease. This is demonstrated by the fact that ER-negative tumors did not show a correlation. The correlation reported in Hu only indicates that the greater the change in expression level, the more likely it is that there is a *published* or *known* role for the gene in the disease, as found by their automated literature-mining software. Nowhere in Hu does it say that a lack of correlation in their study means that the genes, and their corresponding proteins, with a less than five-fold change in level of expression in cancer cannot serve as a molecular marker of cancer. Genes with lower levels of change in expression may or may not be the most important genes in causing the disease, but the genes and their corresponding proteins can still show a consistent and measurable change in expression. While such genes and polypeptides may or may not be good targets for further research, they can nonetheless be used as diagnostic tools. Thus, Hu does not refute the Applicants' assertion that the PRO1357 polypeptide, and its encoding nucleic acid, can be used as a cancer diagnostic tool because they are differentially expressed in certain tumors.

The PTO also relies on Haynes et al. (1998, Electrophoresis 19:1862-1871) and Konopka et al. (1986, PNAS 83:4049-4052) to attempt to refute the general rule that there is a correlation between nucleic acid expression and protein expression. The references do not support the PTO's position or refute the utility of the claims.

Haynes is a review article dealing with the art of proteome analysis. The assertions in Haynes cited by the Examiner were made in an effort to identify shortcomings in the art of mRNA quantification to argue for "proteome analysis to become an essential component in the comprehensive analysis of biological systems." Haynes, p. 1863. Haynes studied 80 selected

Appl. No. : 10/063,587
Filed : May 3, 2002

samples from *Saccharomyces cerevisiae*, and reported “a general trend but no strong correlation between protein and transcript levels (Fig. 1).” *Id.* However, a cursory inspection of Fig. 1 shows a clear correlation between the mRNA levels and protein levels measured. This correlation is confirmed by an inspection of the full-length research paper from which the data in Fig. 1 were derived, presented herein as Exhibit 5 (Gygi et al., Molecular and Cellular Biology, Mar. 1999, 1720-1730). Gygi states that “there was a general trend of increased protein levels resulting from increased mRNA levels,” with a correlation coefficient of 0.935, indicating a strong correlation. Gygi, p. 1726. Moreover, Gygi also states that the correlation is especially strong for highly expressed mRNAs. *Id.* Considering that Example 18 of the specification shows higher expression of PRO1357 mRNA in normal stomach and normal lung tissue as compared to stomach tumor and lung tumor, Haynes and Gygi actually provide strong evidence in support of a general correlation between mRNA and protein levels, and thus further support the utility of the PRO1357 polypeptides and the claimed antibodies to the same.

The 50-fold variation referred to by Haynes and cited by the Examiner, does not in any way show the absence of a correlation between mRNA and protein levels, but rather identifies the outer limits of variability in the authors’ experiments. This variability may support the authors’ assertion that the amount of a particular protein cannot accurately predict the particular level of the corresponding mRNA transcript, but it does not suggest an absence of a general correlation between mRNA and protein levels. Again, Applicants’ utility is based on the differential expression of mRNA in normal stomach and lung versus stomach tumor and lung tumor. Exact levels of expression are irrelevant. Moreover, Gygi states that the high degree of variability seen at low levels of mRNA (shown in inset of Fig. 1, Haynes p. 1863) is due to the fact that “the magnitude of the error in the measurement of mRNA levels is inversely proportional to the mRNA levels.” Gygi, p. 1727. Considering that PRO1357 mRNA has been shown in Example 18 of the specification to be more highly expressed in normal stomach tissue than stomach tumor, and in normal lung tissue than in lung tumor, the variability identified by Haynes is even less applicable to establishing the absence of a correlation between mRNA and protein levels in the instant case.

As stated above, the standard for utility is not absolute certainty, but rather whether one of skill in the art would be more likely than not to believe the asserted utility. Here, the utility requirement does not require Applicants to show that mRNA levels correlate to protein levels in

Appl. No. : 10/063,587
Filed : May 3, 2002

every case, but rather only that the correlation exists more often than not. The data presented in Haynes is not inconsistent with or contradictory to the utility or enablement of the instant claims. To the contrary, the data clearly show a general correlation between protein levels and mRNA levels, and thus support Applicants' assertion that such a general correlation exists.

Even if Haynes supported the Examiner's argument, which it does not, one contrary example does not establish that one of skill in the art would find it is more likely than not there is no general correlation between mRNA level and protein levels. In fact, the working hypothesis among those skilled in the art, as illustrated by the evidence presented above by Applicants, is that there is a direct correlation between mRNA levels and protein levels. This is further supported by the statement in Haynes that "interpretations of quantitative mRNA expression profiles frequently implicitly or explicitly assume that for specific genes the transcript levels are indicative of the levels of protein expression." See, Haynes, p. 1863, first full paragraph. Haynes does not suggest there is no correlation between mRNA and protein levels, but rather points to what the authors believe are shortcomings of using mRNA quantification to predict protein levels; specifically, that mRNA levels may not accurately predict protein levels *in each particular instance*. Considering the more likely than not standard for utility, Haynes' identification of reasons why proteomic analysis may be preferable in some cases does not contradict Applicants' evidence that there is a general correlation between mRNA and protein levels.

The PTO cites Konopka in further support of the assertion that it is not the norm that levels of mRNA correlate with corresponding protein levels. The PTO has confused the relationship between an increase in copy number of a gene and the level of mRNA on the one hand, with the relationship between mRNA expression and levels of the corresponding protein on the other. In particular, the PTO cites the statement in Konopka that "protein expression is not related to amplification of the abl gene but to variation in the level of the bcr-abl mRNA produced from a single Ph1 template." The results presented in Konopka actually present strong evidence in support of Applicants' position that there is a general understanding in the art that levels of mRNA correlate with levels of the corresponding proteins. Konopka analyzed the expression patterns of a gene associated with certain cancers. The authors show a wide variation in the levels of the protein in various cell types, and find that this variation can be attributed to the levels of the corresponding mRNA in each cell type. See, Konopka at 4050. Konopka thus

Appl. No. : 10/063,587
Filed : May 3, 2002

concludes, "these combined data suggest that differential bcr-abl mRNA expression from a single gene template is responsible for the variable levels of P210^{c-abl} [the protein of interest] detected." *Id.* at. 4051. Thus, far from supporting the PTO's assertion that it is not the norm that increased transcription leads to increased levels of the corresponding protein, Konopka strongly supports the opposite proposition asserted by Applicants - that the level of mRNA, more often than not, correlates with the level of the corresponding protein.

Thus, the references cited by the PTO as evidence that no such general understanding exists do not in any way support the PTO's arguments. To the contrary, Haynes and Konopka each provide strong evidence of the correlation asserted by Applicants. Accordingly, Applicants respectfully submit that the totality of the evidence clearly supports the conclusion that one of skill in the art understands that, more likely than not, levels of mRNA directly correlate with levels of corresponding proteins.

Together, the declarations of Grimaldi and Polakis, the accompanying references, as well as the other submitted excerpts and literature references, all establish that the accepted understanding in the art is that there is a reasonable correlation between changes in gene expression and the level of the encoded protein. Despite some teachings in the art of certain genes that do not fit within this paradigm which are exceptions rather than the rule, in the vast majority of cases, the combined teachings in the art, exemplified by cited references and the Grimaldi and Polakis declarations, overwhelmingly teach that gene expression influences mRNA expression and protein levels. Considering that utility does not have to be proven to an absolute certainty, Applicants submit they have provided sufficient evidence to show a reasonable correlation between mRNA expression and the level of PRO1357 protein. In light of the lack of support for any argument by the PTO to the contrary, Applicants submit that they have established that it is more likely than not that one of skill in the art would believe that because the PRO1357 mRNA is more highly expressed in normal stomach tissue and normal lung tissue compared to stomach tumor tissue and lung tumor tissue, respectively, the PRO1357 polypeptide will also be more highly expressed in normal stomach tissue and normal lung tissue compared to stomach tumor tissue and lung tumor tissue, respectively. One of skill in the art would recognize that a nucleic acid or polypeptide which is differentially expressed in certain cancer cells compared to the corresponding normal tissue would have utility as a diagnostic tool to screen between normal and tumor tissue samples. Thus, Applicants submit that they have established

Appl. No. : **10/063,587**
Filed : **May 3, 2002**

that it is more likely than not that one of skill in the art would recognize the asserted utility of the differentially expressed nucleic acids, the PRO1357 polypeptides, and the claimed antibodies as diagnostic tools for both stomach and lung tumors.

Conclusion

As set forth above, Applicants have established that the ordinary skilled artisan recognizes the sufficiency of the correlation between the differential expression data in Example 18 and use in distinguishing between cell types (cancerous versus non-cancerous) so as to convince the skilled artisan to a reasonable probability that the claimed antibodies are useful as a screening tool in cancer diagnostics. Applicants have rebutted all of the arguments set forth in the Final Office Action regarding this matter.

Also, Applicants have provided additional evidence in support of the correlation between DNA expression and protein expression.

Thus, given the totality of the evidence provided, Applicants submit that they have established a substantial, specific, and credible utility for the nucleic acids, the encoded PRO1357 polypeptides, and the claimed antibodies as diagnostic agents. According to the PTO Utility Examination Guidelines (2001), irrefutable proof of a claimed utility is not required. Rather, a specific, substantial, and credible utility requires only a “reasonable” confirmation of a real world context of use. Applicants submit that they have established that it is more likely than not that one of skill in the art would reasonably accept the utility for the claimed antibodies set forth in the specification. Applicants believe that they have met their burden of establishing a specific and substantial credible utility for the claimed invention.

In view of the above, Applicants respectfully request that the PTO reconsider and withdraw the utility rejection under 35 U.S.C. §101.

Rejection under 35 U.S.C. §112, first paragraph – Enablement

The PTO has maintained the rejection of Claims 1-5 as lacking enablement under 35 U.S.C. § 112, first paragraph. According to the Examiner, because the claimed invention is not supported by either a substantial asserted utility or a well established utility, one of skill in the art would not know how to use the invention.

Appl. No. : **10/063,587**
Filed : **May 3, 2002**

Applicants believe that the evidence, declarations, references, and arguments discussed above make clear that Applicants have established that one of skill in the art would be convinced, to a reasonable probability, that the PRO1357 polypeptides are overexpressed in normal stomach tissue and normal lung tissue, and that therefore antibodies to those polypeptides have utility as diagnostic tools for screening tissue to detect stomach and lung tumors. Diagnostic and therapeutic antibodies can be created that bind to the encoded PRO1357 polypeptides. This is disclosed in the application, and the techniques for the creation of antibodies are well known and routine in the art. Thus, at least one use of PRO1357 nucleic acids, the encoded polypeptides and the claimed antibodies is adequately enabled, which is all that is required – “if any use is enabled when multiple uses are disclosed, the application is enabling for the claimed invention.” M.P.E.P. 2164.01(c). In view of the above, Applicants respectfully request that the Examiner reconsider and withdraw the enablement rejection under 35 U.S.C. § 112, first paragraph.

Rejection under 35 U.S.C. §102(b) – Anticipation

Claims 1-5 remain rejected under 35 U.S.C. § 102(b) as being anticipated by WO 01/16318 and WO 00/12708.

The data in Example 18 were disclosed in priority application, PCT/US00/23328 filed August 24, 2000, which is the PCT application published as WO 01/16318. As discussed above, the instant claimed subject matter has utility based upon the data in Example 18 and the instant application is a continuation of PCT/US00/23328; therefore, the present claims are entitled to the filing date of August 24, 2000. WO 01/16318 is not prior art under § 102(b).

WO 00/12708 was published on March 9, 2000, which is less than one year before the filing of priority application PCT/US00/23328 (August 24, 2000). Again, PCT/US00/23328 discloses the differential expression data which provides utility for the instant claims, and Applicants are entitled to the filing date of August 24, 2000. Therefore, WO 00/12708 cannot be cited under § 102(b).

In view of the above discussion, reconsideration and withdrawal of the rejection under § 102(b) is respectfully requested.

Appl. No. : 10/063,587
Filed : May 3, 2002

CONCLUSION

In view of the above, Applicants respectfully maintain that claims are patentable and request that they be passed to issue. Applicants invite the Examiner to call the undersigned if any remaining issues may be resolved by telephone.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 2/10/05

By: M. T. Morley
Marc T. Morley
Registration No. 52,051
Attorney of Record
Customer No. 30,313
(619) 235-8550

S:\DOCS\MTM\MTM-7654.DOC
020705

MOLECULAR BIOLOGY OF THE CELL

fourth edition

Bruce Alberts

Alexander Johnson

Julian Lewis

Martin Raff

Keith Roberts

Peter Walter

GS Garland Science
Taylor & Francis Group

Garland

Vice President: Denise Schanck
Managing Editor: Sarah Gibbs
Senior Editorial Assistant: Kirsten Jenner
Managing Production Editor: Emma Hunt
Proofreader and Layout: Emma Hunt
Production Assistant: Angela Bennett
Text Editors: Marjorie Singer Anderson and Betsy Dilemia
Copy Editor: Bruce Goatly
Word Processors: Fran Dependahl, Misty Landers and Carol Winter
Designer: Blink Studio, London
Illustrator: Nigel Orme
Indexer: Janine Ross and Sherry Granum
Manufacturing: Nigel Eyre and Marion Morrow

Cell Biology Interactive

Artistic and Scientific Direction: Peter Walter
Narrated by: Julie Theriot
Production, Design, and Development: Mike Morales

Bruce Alberts received his Ph.D. from Harvard University and is President of the National Academy of Sciences and Professor of Biochemistry and Biophysics at the University of California, San Francisco. Alexander Johnson received his Ph.D. from Harvard University and is a Professor of Microbiology and Immunology at the University of California, San Francisco. Julian Lewis received his D.Phil. from the University of Oxford and is a Principal Scientist at the Imperial Cancer Research Fund, London. Martin Raff received his M.D. from McGill University and is at the Medical Research Council Laboratory for Molecular Cell Biology and Cell Biology Unit and in the Biology Department at University College London. Keith Roberts received his Ph.D. from the University of Cambridge and is Associate Research Director at the John Innes Centre, Norwich. Peter Walter received his Ph.D. from The Rockefeller University in New York and is Professor and Chairman of the Department of Biochemistry and Biophysics at the University of California, San Francisco, and an investigator of the Howard Hughes Medical Institute.

© 2002 by Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter.
© 1983, 1989, 1994 by Bruce Alberts, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts, and James D. Watson.

All rights reserved. No part of this book covered by the copyright hereon may be reproduced or used in any format in any form or by any means—graphic, electronic, or mechanical, including photocopying, recording, taping, or information storage and retrieval systems—without permission of the publisher.

Library of Congress Cataloging-in-Publication Data
Molecular biology of the cell / Bruce Alberts ... [et al.]. -- 4th ed.
p. cm
Includes bibliographical references and index.
ISBN 0-8153-3218-1 (hardbound) -- ISBN 0-8153-4072-9 (pbk.)
1. Cytology. 2. Molecular biology. I. Alberts, Bruce.
[DNLM: 1. Cells. 2. Molecular Biology.]
QH581.2 .M64 2002
571.6--dc21

2001054471 CIP

Published by Garland Science, a member of the Taylor & Francis Group,
29 West 35th Street, New York, NY 10001-2299

Printed in the United States of America

15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

Front cover Human Genome: Reprinted by permission from *Nature*, International Human Genome Sequencing Consortium, 409:860-921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila*, *Arabidopsis*, Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all." (Gunter Blobel, courtesy of The Rockefeller University; Marie Curie, Keystone Press Agency Inc; Darwin bust, by permission of the President and Council of the Royal Society; Rosalind Franklin, courtesy of Cold Spring Harbor Laboratory Archives; Dorothy Hodgkin, © The Nobel Foundation, 1964; James Joyce, etching by Peter Blake; Robert Johnson, photo booth self-portrait early 1930s, © 1986 Delta Haze Corporation all rights reserved, used by permission; Albert L. Lehninger, (unidentified photographer) courtesy of The Alan Mason Chesney Medical Archives of The Johns Hopkins Medical Institutions; Linus Pauling, from Ava Helen and Linus Pauling Papers, Special Collections, Oregon State University; Nicholas Poussin, courtesy of ArtToday.com; Barbara McClintock, © David Micklos, 1983; Andrei Sakharov, courtesy of Elena Bonner; Frederick Sanger, © The Nobel Foundation, 1958.)

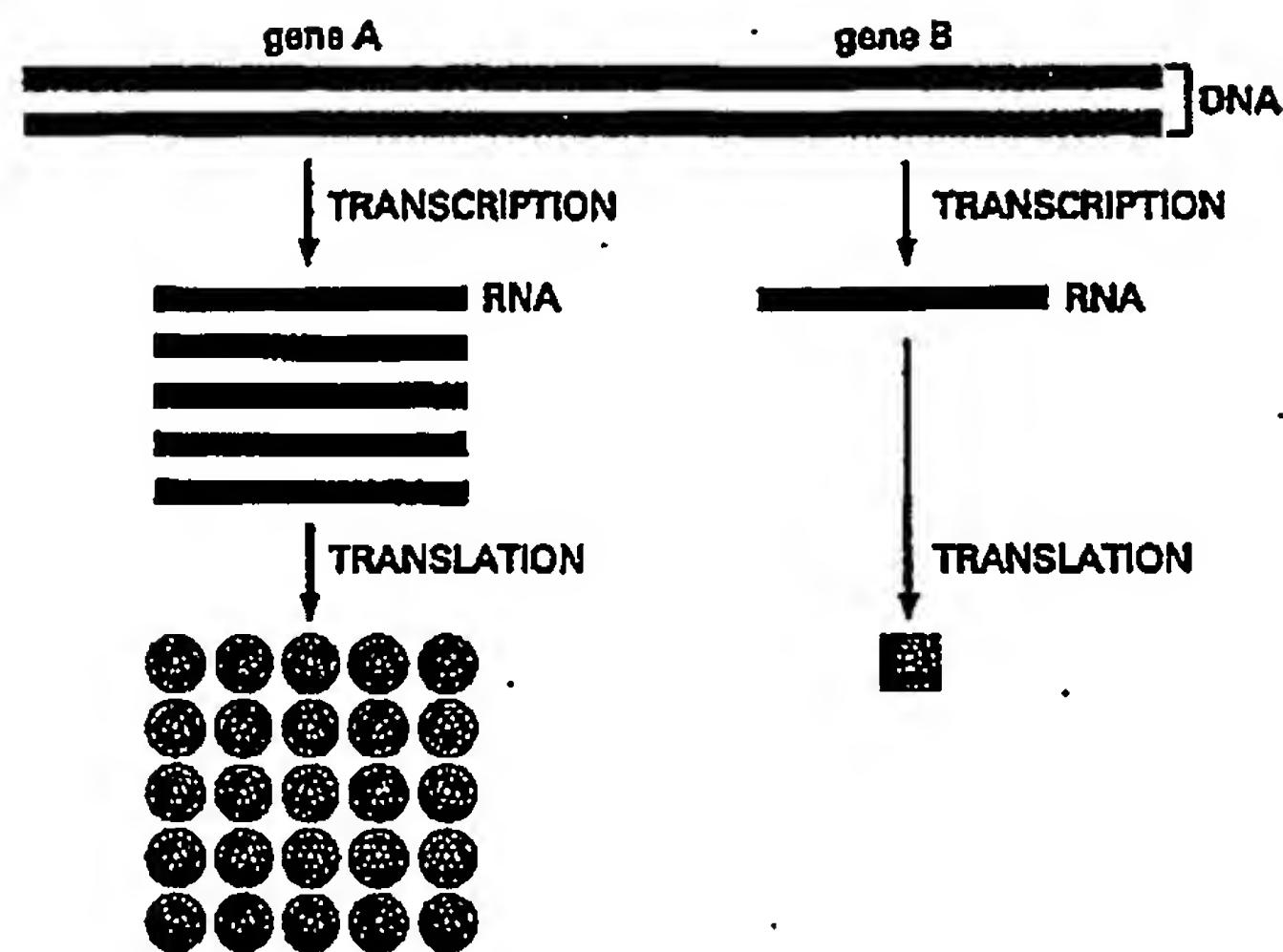


Figure 6-3 Genes can be expressed with different efficiencies. Gene A is transcribed and translated much more efficiently than gene B. This allows the amount of protein A in the cell to be much greater than that of protein B.

FROM DNA TO RNA

Transcription and translation are the means by which cells read out, or express, the genetic instructions in their genes. Because many identical RNA copies can be made from the same gene, and each RNA molecule can direct the synthesis of many identical protein molecules, cells can synthesize a large amount of protein rapidly when necessary. But each gene can also be transcribed and translated with a different efficiency, allowing the cell to make vast quantities of some proteins and tiny quantities of others (Figure 6-3). Moreover, as we see in the next chapter, a cell can change (or regulate) the expression of each of its genes according to the needs of the moment—most obviously by controlling the production of its RNA.

Portions of DNA Sequence Are Transcribed into RNA

The first step a cell takes in reading out a needed part of its genetic instructions is to copy a particular portion of its DNA nucleotide sequence—a gene—into an RNA nucleotide sequence. The information in RNA, although copied into another chemical form, is still written in essentially the same language as it is in DNA—the language of a nucleotide sequence. Hence the name **transcription**.

Like DNA, RNA is a linear polymer made of four different types of nucleotide subunits linked together by phosphodiester bonds (Figure 6-4). It differs from DNA chemically in two respects: (1) the nucleotides in RNA are *ribonucleotides*—that is, they contain the sugar ribose (hence the name *ribonucleic acid*) rather than deoxyribose; (2) although, like DNA, RNA contains the bases adenine (A), guanine (G), and cytosine (C), it contains the base uracil (U) instead of the thymine (T) in DNA. Since U, like T, can base-pair by hydrogen-bonding with A (Figure 6-5), the complementary base-pairing properties described for DNA in Chapters 4 and 5 apply also to RNA (in RNA, G pairs with C, and A pairs with U). It is not uncommon, however, to find other types of base pairs in RNA: for example, G pairing with U occasionally.

Despite these small chemical differences, DNA and RNA differ quite dramatically in overall structure. Whereas DNA always occurs in cells as a double-stranded helix, RNA is single-stranded. RNA chains therefore fold up into a variety of shapes, just as a polypeptide chain folds up to form the final shape of a protein (Figure 6-6). As we see later in this chapter, the ability to fold into complex three-dimensional shapes allows some RNA molecules to have structural and catalytic functions.

Transcription Produces RNA Complementary to One Strand of DNA

All of the RNA in a cell is made by DNA transcription, a process that has certain similarities to the process of DNA replication discussed in Chapter 5.

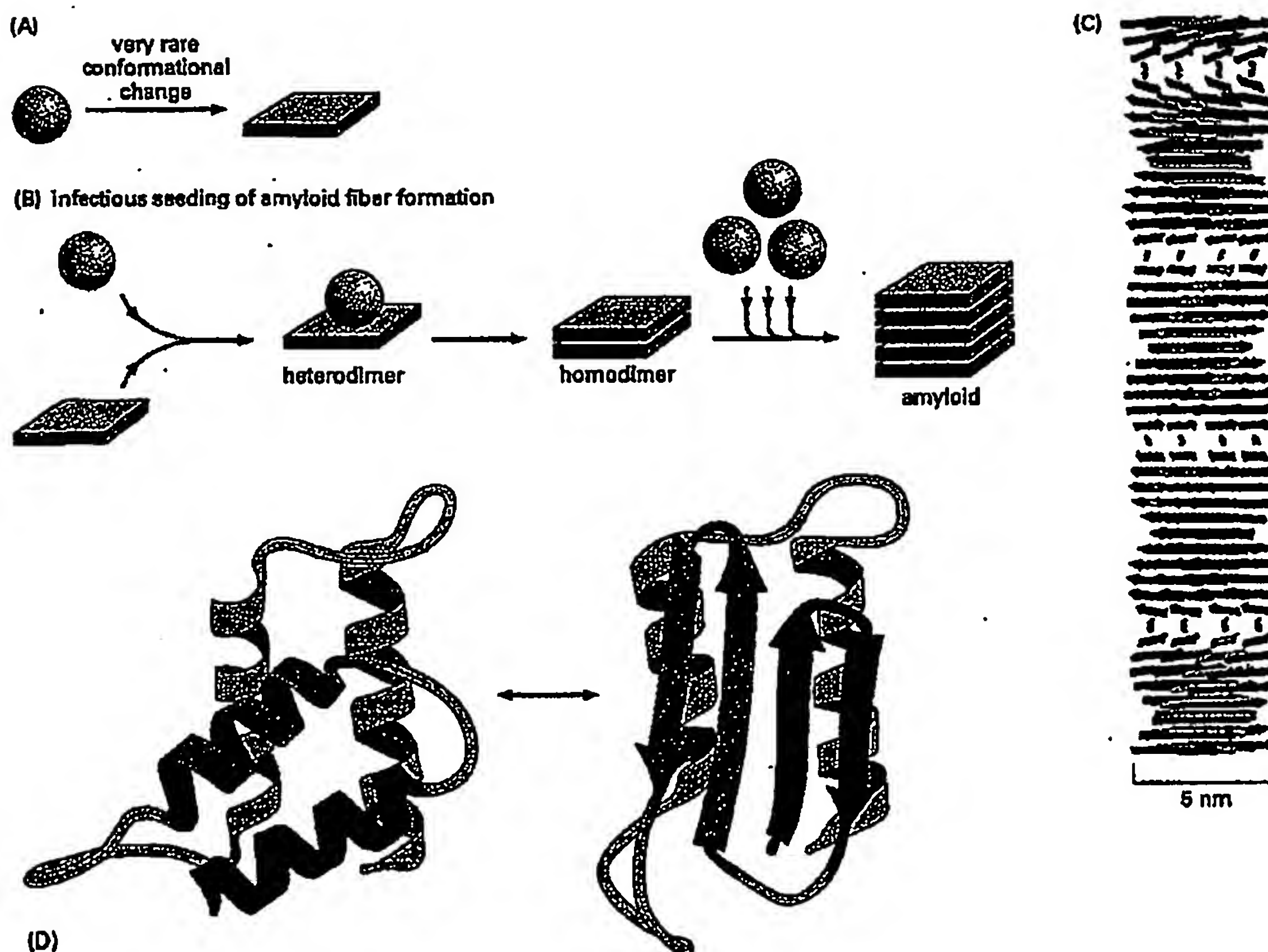


Figure 6-89 Protein aggregates that cause human disease. (A) Schematic illustration of the type of conformational change in a protein that produces material for a cross-beta filament. (B) Diagram illustrating the self-infectious nature of the protein aggregation that is central to prion diseases. PrP is highly unusual because the misfolded version of the protein, called PrP^{*}, induces the normal PrP protein it contacts to change its conformation, as shown. Most of the human diseases caused by protein aggregation are caused by the overproduction of a variant protein that is especially prone to aggregation, but because this structure is not infectious in this way, it cannot spread from one animal to another. (C) Drawing of a cross-beta filament, a common type of protease-resistant protein aggregate found in a variety of human neurological diseases. Because the hydrogen-bond interactions in a β sheet form between polypeptide backbone atoms (see Figure 3-9), a number of different abnormally folded proteins can produce this structure. (D) One of several possible models for the conversion of PrP to PrP^{*}, showing the likely change of two α -helices into four β -strands. Although the structure of the normal protein has been determined accurately, the structure of the infectious form is not yet known with certainty because the aggregation has prevented the use of standard structural techniques. (C, courtesy of Louise Serpell, adapted from M. Sunde et al., *J. Mol. Biol.* 273:729-739, 1997; D, adapted from S.B. Prusiner, *Trends Biochem. Sci.* 21:482-487, 1996.)

animals and humans. It can be dangerous to eat the tissues of animals that contain PrP^{*}, as witnessed most recently by the spread of BSE (commonly referred to as the "mad cow disease") from cattle to humans in Great Britain.

Fortunately, in the absence of PrP^{*}, PrP is extraordinarily difficult to convert to its abnormal form. Although very few proteins have the potential to misfold into an infectious conformation, a similar transformation has been discovered to be the cause of an otherwise mysterious "protein-only inheritance" observed in yeast cells.

There Are Many Steps From DNA to Protein

We have seen so far in this chapter that many different types of chemical reactions are required to produce a properly folded protein from the information contained in a gene (Figure 6-90). The final level of a properly folded protein in a cell therefore depends upon the efficiency with which each of the many steps is performed.

We discuss in Chapter 7 that cells have the ability to change the levels of their proteins according to their needs. In principle, any or all of the steps in Fig-

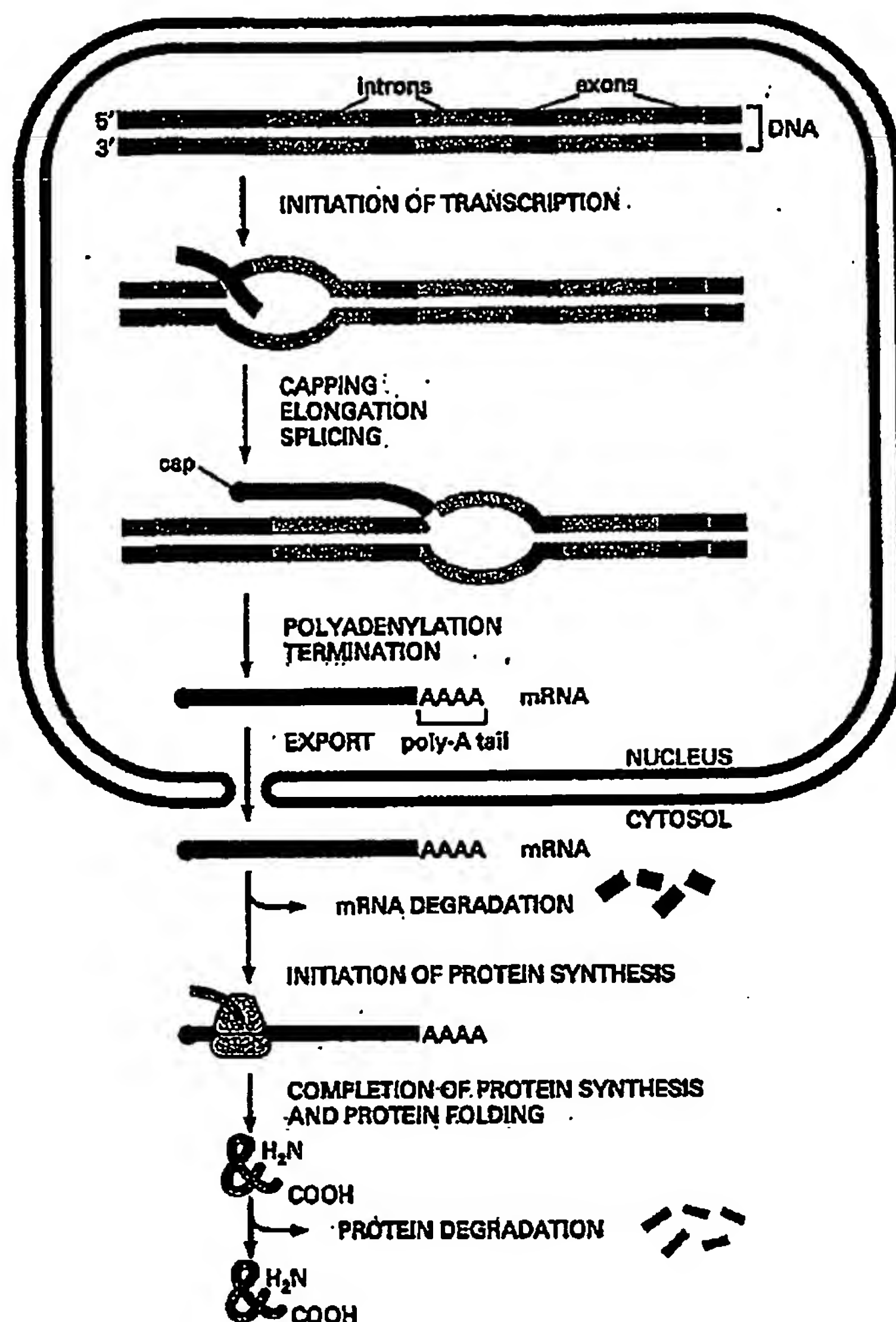


Figure 6-90 The production of a protein by a eucaryotic cell. The final level of each protein in a eucaryotic cell depends upon the efficiency of each step depicted.

ure 6-90) could be regulated by the cell for each individual protein. However, as we shall see in Chapter 7, the initiation of transcription is the most common point for a cell to regulate the expression of each of its genes. This makes sense, inasmuch as the most efficient way to keep a gene from being expressed is to block the very first step—the transcription of its DNA sequence into an RNA molecule.

Summary

The translation of the nucleotide sequence of an mRNA molecule into protein takes place in the cytoplasm on a large ribonucleoprotein assembly called a ribosome. The amino acids used for protein synthesis are first attached to a family of tRNA molecules, each of which recognizes, by complementary base-pair interactions, particular sets of three nucleotides in the mRNA (codons). The sequence of nucleotides in the mRNA is then read from one end to the other in sets of three according to the genetic code.

To initiate translation, a small ribosomal subunit binds to the mRNA molecule at a start codon (AUG) that is recognized by a unique initiator tRNA molecule. A large ribosomal subunit binds to complete the ribosome and begin the elongation phase of protein synthesis. During this phase, aminoacyl tRNAs—each bearing a specific amino acid bind sequentially to the appropriate codon in mRNA by forming complementary base pairs with the tRNA anticodon. Each amino acid is added to the C-terminal end of the growing polypeptide by means of a cycle of three sequential

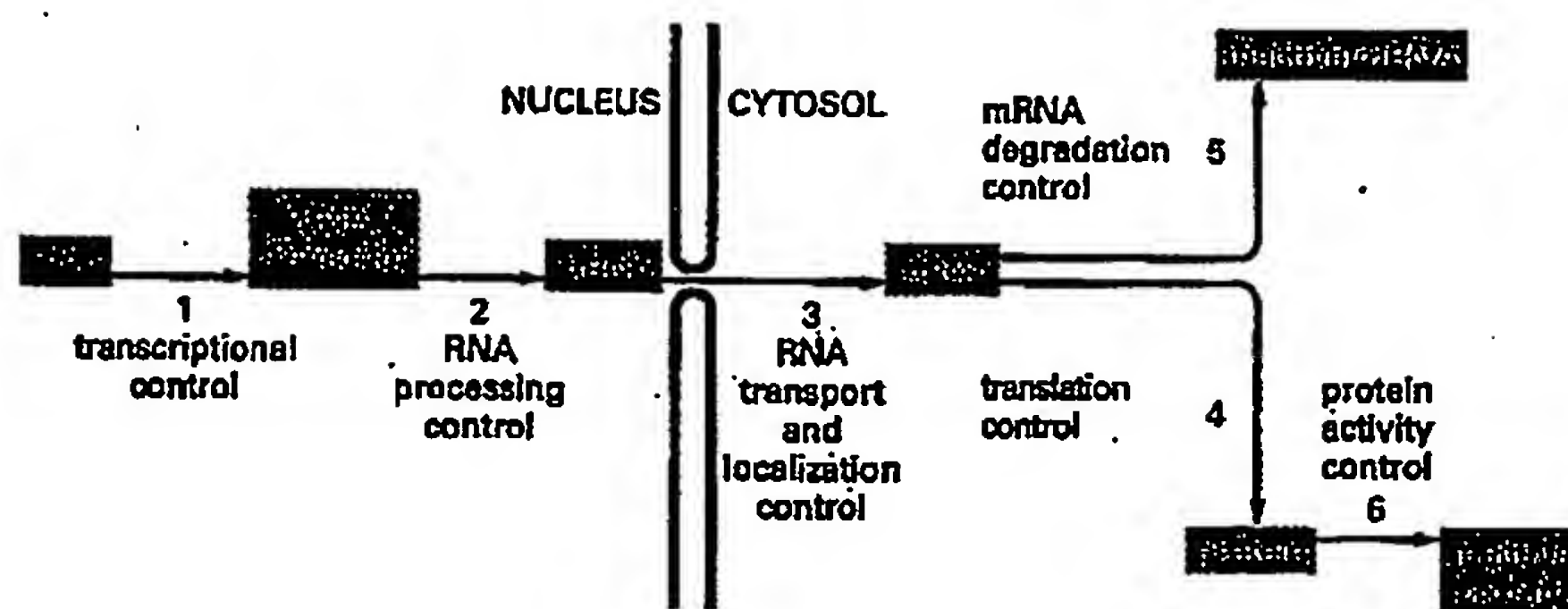


Figure 7-5 Six steps at which eucaryotic gene expression can be controlled. Controls that operate at steps 1 through 5 are discussed in this chapter. Step 6, the regulation of protein activity, includes reversible activation or inactivation by protein phosphorylation (discussed in Chapter 3) as well as irreversible inactivation by proteolytic degradation (discussed in Chapter 6).

Gene Expression Can Be Regulated at Many of the Steps in the Pathway from DNA to RNA to Protein

If differences among the various cell types of an organism depend on the particular genes that the cells express, at what level is the control of gene expression exercised? As we saw in the last chapter, there are many steps in the pathway leading from DNA to protein, and all of them can in principle be regulated. Thus a cell can control the proteins it makes by (1) controlling when and how often a given gene is transcribed (**transcriptional control**), (2) controlling how the RNA transcript is spliced or otherwise processed (**RNA processing control**), (3) selecting which completed mRNAs in the cell nucleus are exported to the cytosol and determining where in the cytosol they are localized (**RNA transport and localization control**), (4) selecting which mRNAs in the cytoplasm are translated by ribosomes (**translational control**), (5) selectively destabilizing certain mRNA molecules in the cytoplasm (**mRNA degradation control**), or (6) selectively activating, inactivating, degrading, or compartmentalizing specific protein molecules after they have been made (**protein activity control**) (Figure 7-5).

For most genes transcriptional controls are paramount. This makes sense because, of all the possible control points illustrated in Figure 7-5, only transcriptional control ensures that the cell will not synthesize superfluous intermediates. In the following sections we discuss the DNA and protein components that perform this function by regulating the initiation of gene transcription. We shall return at the end of the chapter to the additional ways of regulating gene expression.

Summary

The genome of a cell contains in its DNA sequence the information to make many thousands of different protein and RNA molecules. A cell typically expresses only a fraction of its genes, and the different types of cells in multicellular organisms arise because different sets of genes are expressed. Moreover, cells can change the pattern of genes they express in response to changes in their environment, such as signals from other cells. Although all of the steps involved in expressing a gene can in principle be regulated, for most genes the initiation of RNA transcription is the most important point of control.

DNA-BINDING MOTIFS IN GENE REGULATORY PROTEINS

How does a cell determine which of its thousands of genes to transcribe? As mentioned briefly in Chapters 4 and 6, the transcription of each gene is controlled by a regulatory region of DNA relatively near the site where transcription begins. Some regulatory regions are simple and act as switches that are thrown by a single signal. Many others are complex and act as tiny microprocessors, responding to a variety of signals that they interpret and integrate to switch the neighboring gene on or off. Whether complex or simple, these switching devices

occur in the germ line, the cell lineage that gives rise to sperm or eggs. Most of the DNA in vertebrate germ cells is inactive and highly methylated. Over long periods of evolutionary time, the methylated CG sequences in these inactive regions have presumably been lost through spontaneous deamination events that were not properly repaired. However promoters of genes that remain active in the germ cell lineages (including most housekeeping genes) are kept unmethylated, and therefore spontaneous deaminations of Cs that occur within them can be accurately repaired. Such regions are preserved in modern day vertebrate cells as CG islands. In addition, any mutation of a CG sequence in the genome that destroyed the function or regulation of a gene in the adult would be selected against, and some CG islands are simply the result of a higher than normal density of critical CG sequences.

The mammalian genome contains an estimated 20,000 CG islands. Most of the islands mark the 5' ends of transcription units and thus, presumably, of genes. The presence of CG islands often provides a convenient way of identifying genes in the DNA sequences of vertebrate genomes.

Summary

The many types of cells in animals and plants are created largely through mechanisms that cause different genes to be transcribed in different cells. Since many specialized animal cells can maintain their unique character through many cell division cycles and even when grown in culture, the gene regulatory mechanisms involved in creating them must be stable once established and heritable when the cell divides. These features endow the cell with a memory of its developmental history. Bacteria and yeasts provide unusually accessible model systems in which to study gene regulatory mechanisms. One such mechanism involves a competitive interaction between two gene regulatory proteins, each of which inhibits the synthesis of the other; this can create a flip-flop switch that switches a cell between two alternative patterns of gene expression. Direct or indirect positive feedback loops, which enable gene regulatory proteins to perpetuate their own synthesis, provide a general mechanism for cell memory. Negative feedback loops with programmed delays form the basis for cellular clocks.

In eucaryotes the transcription of a gene is generally controlled by combinations of gene regulatory proteins. It is thought that each type of cell in a higher eucaryotic organism contains a specific combination of gene regulatory proteins that ensures the expression of only those genes appropriate to that type of cell. A given gene regulatory protein may be active in a variety of circumstances and typically is involved in the regulation of many genes.

In addition to diffusible gene regulatory proteins, inherited states of chromatin condensation are also used by eucaryotic cells to regulate gene expression. An especially dramatic case is the inactivation of an entire X chromosome in female mammals. In vertebrates DNA methylation also functions in gene regulation, being used mainly as a device to reinforce decisions about gene expression that are made initially by other mechanisms. DNA methylation also underlies the phenomenon of genomic imprinting in mammals, in which the expression of a gene depends on whether it was inherited from the mother or the father.

POSTTRANSCRIPTIONAL CONTROLS

In principle, every step required for the process of gene expression could be controlled. Indeed, one can find examples of each type of regulation, although any one gene is likely to use only a few of them. Controls on the initiation of gene transcription are the predominant form of regulation for most genes. But other controls can act later in the pathway from DNA to protein to modulate the amount of gene product that is made. Although these posttranscriptional controls, which operate after RNA polymerase has bound to the gene's promoter and begun RNA synthesis, are less common than transcriptional control, for many genes they are crucial.

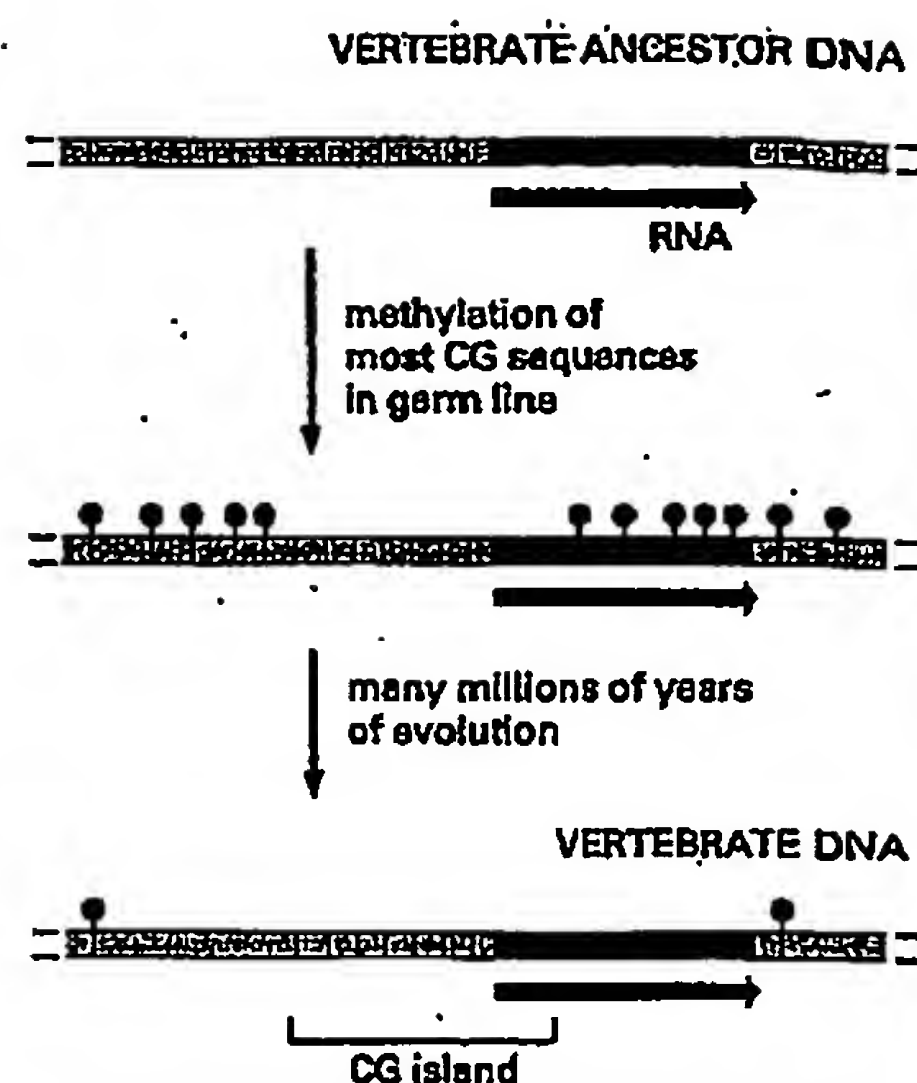


Figure 7-86 A mechanism to explain both the marked overall deficiency of CG sequences and their clustering into CG islands in vertebrate genomes. A black line marks the location of a CG dinucleotide in the DNA sequence, while a red "lollipop" indicates the presence of a methyl group on the CG dinucleotide. CG sequences that lie in regulatory sequences of genes that are transcribed in germ cells are unmethylated and therefore tend to be retained in evolution. Methylated CG sequences, on the other hand, tend to be lost through deamination of 5-methyl C to T, unless the CG sequence is critical for survival.

CHAPTER 29

Regulation of transcription

Genes VI (1997) CH 29, pp. 847-848.
Benjamin Lewin

The phenotypic differences that distinguish the various kinds of cells in a higher eukaryote are largely due to differences in the expression of genes that code for proteins, that is, those transcribed by RNA polymerase II. In principle, the expression of these genes might be regulated at any one of several stages. The concept of the "level of control" implies that gene expression is not necessarily an automatic process once it has begun. It could be regulated in a gene-specific way at any one of several sequential steps. We can distinguish (at least) five potential control points, forming the series:

Activation of gene structure
↓
Initiation of transcription
↓
Processing the transcript
↓
Transport to cytoplasm
↓
Translation of mRNA

The existence of the first step is implied by the discovery that genes may exist in either of two structural conditions. Relative to the state of most of the genome, genes are found in an "active" state in the cells in which they are expressed (see Chapter 27). The change of structure is distinct from the act of transcription, and indicates that the gene is "transcribable." This suggests that acquisition of the "active" structure must be the first step in gene expression.

Transcription of a gene in the active state is

controlled at the stage of initiation, that is, by the interaction of RNA polymerase with its promoter. This is now becoming susceptible to analysis in the *in vitro* systems (see Chapter 28). For most genes, this is a major control point; probably it is the most common level of regulation.

There is at present no evidence for control at subsequent stages of transcription in eukaryotic cells, for example, via antitermination mechanisms.

The primary transcript is modified by capping at the 5' end, and usually also by polyadenylation at the 3' end. Introns must be spliced out from the transcripts of interrupted genes. The mature RNA must be exported from the nucleus to the cytoplasm. Regulation of gene expression by selection of sequences at the level of nuclear RNA might involve any or all of these stages, but the one for which we have most evidence concerns changes in splicing: some genes are expressed by means of alternative splicing patterns whose regulation controls the type of protein product (see Chapter 30).

Finally, the translation of an mRNA in the cytoplasm can be specifically controlled. There is little evidence for the employment of this mechanism in adult somatic cells, but it does occur in some embryonic situations, as described in Chapter 7. The mechanism is presumed to involve the blocking of initiation of translation of some mRNAs by specific protein factors.

But having acknowledged that control of gene expression can occur at multiple stages, and that production of RNA cannot inevitably be equated with production of protein, it is clear

that the overwhelming majority of regulatory events occur at the initiation of transcription. Regulation of tissue-specific gene transcription lies at the heart of eukaryotic differentiation; indeed, we see examples in Chapter 38 in which proteins that regulate embryonic development prove to be transcription factors. A regulatory transcription factor serves to provide

common control of a large number of target genes, and we seek to answer two questions about this mode of regulation: what identifies the common target genes to the transcription factor, and how is the activity of the transcription factor itself regulated in response to intrinsic or extrinsic signals?

Response elements identify genes under common regulation

The principle that emerges from characterizing groups of genes under common control is that *they share a promoter element that is recognized by a regulatory transcription factor*. An element that causes a gene to respond to such a factor is called a response element; examples are the HSE (heat shock response element), GRE (glucocorticoid response element), SRE (serum response element).

The properties of some inducible transcription factors and the elements that they recognize are summarized in Table 29.1. Response elements have the same general characteristics as upstream elements of promoters or enhancers. They contain short consensus sequences, and copies of the response elements found in different genes are closely related, but not necessarily identical. The region bound by the factor extends for a short distance on either side of

the consensus sequence. In promoters, the elements are not present at fixed distances from the startpoint, but are usually <200 bp upstream of it. The presence of a single element usually is sufficient to confer the regulatory response, but sometimes there are multiple copies.

Response elements may be located in promoters or in enhancers. Some types of elements are typically found in one rather than the other: usually an HSE is found in a promoter, while a GRE is found in an enhancer. We assume that all response elements function by the same general principle. A gene is regulated by a sequence at the promoter or enhancer that is recognized by a specific protein. The protein functions as a transcription factor needed for RNA polymerase to initiate. Active protein is available only under conditions when the gene is to be expressed; its absence means that the promoter is not activated by this particular circuit.

An example of a situation in which many genes are controlled by a single factor is provided by the heat shock response. This is common to a wide range of prokaryotes and eukaryotes and involves multiple controls of gene expression: an increase in temperature turns off transcription of some genes, turns on transcription of the heat shock genes, and causes changes in the translation of mRNAs. The control of the heat shock genes illustrates the differences between prokaryotic and eukaryotic modes of control. In bacteria, a new sigma factor is synthesized that directs RNA polymerase holoenzyme to recognize an alternative

Table 29.1 Inducible transcription factors bind to response elements that identify groups of promoters or enhancers subject to coordinate control.

Regulatory Agent	Module	Consensus	Factor
Heat shock	HSE	CNNGAANNTCGNG	HSTF
Glucocorticoid	GRE	TGGTACAAATGTTCT	Receptor
Phorbol ester	TRE	TGACTCA	AP1
Serum	SRE	CCATATTAGG	SRF

Research

Open Access

Prostate stem cell antigen (PSCA) expression in human prostate cancer tissues and its potential role in prostate carcinogenesis and progression of prostate cancer

Zhao Zhigang*¹ and Shen Wenlv²

Address: ¹Department of Urology, Shantou University Medical College, Shantou, Guangdong, China and ²Department of Urology, No 2. Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong, China

Email: Zhao Zhigang* - zgzhao@163.com; Shen Wenlv - wshen99@hotmail.com

* Corresponding author

Published: 10 May 2004

Received: 30 March 2004

Accepted: 10 May 2004

World Journal of Surgical Oncology 2004, 2:13

This article is available from: <http://www.wjso.com/content/2/1/13>

© 2004 Zhigang and Wenlv; licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.

Abstract

Background: Prostate stem cell antigen (PSCA) is a recently defined homologue of the Thy-1/Ly-6 family of glycosylphosphatidylinositol (GPI)-anchored cell surface antigens. The purpose of the present study was to examine the expression status of PSCA protein and mRNA in clinical specimens of human prostate cancer (Pca) and to validate it as a potential molecular target for diagnosis and treatment of Pca.

Materials and Methods: Immunohistochemical (IHC) and *in situ* hybridization (ISH) analyses of PSCA expression were simultaneously performed on paraffin-embedded sections from 20 benign prostatic hyperplasia (BPH), 20 prostatic intraepithelial neoplasm (PIN) and 48 prostate cancer (Pca) tissues, including 9 androgen-independent prostate cancers. The level of PSCA expression was semiquantitatively scored by assessing both the percentage and intensity of PSCA-positive staining cells in the specimens. Then compared PSCA expression between BPH, PIN and Pca tissues and analysed the correlations of PSCA expression level with pathological grade, clinical stage and progression to androgen-independence in Pca.

Results: In BPH and low grade PIN, PSCA protein and mRNA staining were weak or negative and less intense and uniform than that seen in HGPIN and Pca. There were moderate to strong PSCA protein and mRNA expression in 8 of 11 (72.7%) HGPIN and in 40 of 48 (83.4%) Pca specimens examined by IHC and ISH analyses, with statistical significance compared with BPH (20%) and low grade PIN (22.2%) samples ($p < 0.05$, respectively). The expression level of PSCA increased with high Gleason grade, advanced stage and progression to androgen-independence ($p < 0.05$, respectively). In addition, IHC and ISH staining showed a high degree of correlation between PSCA protein and mRNA overexpression.

Conclusions: Our data demonstrate that PSCA as a new cell surface marker is overexpressed by a majority of human Pca. PSCA expression correlates positively with adverse tumor characteristics, such as increasing pathological grade (poor cell differentiation), worsening clinical stage and androgen-independence, and speculatively with prostate carcinogenesis. PSCA protein overexpression results from upregulated transcription of PSCA mRNA. PSCA may have prognostic utility and may be a promising molecular target for diagnosis and treatment of Pca.

Introduction

Prostate cancer (Pca) is the second leading cause of cancer-related death in American men and is becoming a common cancer increasing in China. Despite recently great progress in the diagnosis and management of localized disease, there continues to be a need for new diagnostic markers that can accurately discriminate between indolent and aggressive variants of Pca. There also continues to be a need for the identification and characterization of potential new therapeutic targets on Pca cells. Current diagnostic and therapeutic modalities for recurrent and metastatic Pca have been limited by a lack of specific target antigens of Pca.

Although a number of prostate-specific genes have been identified (i.e. prostate specific antigen, prostatic acid phosphatase, glandular kallikrein 2), the majority of these are secreted proteins not ideally suited for many immunological strategies. So, the identification of new cell surface antigens is critical to the development of new diagnostic and therapeutic approaches to the management of Pca.

Reiter RE et al [1] reported the identification of prostate stem cell antigen (PSCA), a cell surface antigen that is predominantly prostate specific. The PSCA gene encodes a 123 amino acid glycoprotein, with 30% homology to stem cell antigen 2 (Sca 2). Like Sca-2, PSCA also belongs to a member of the Thy-1/Ly-6 family and is anchored by a glycosylphosphatidylinositol (GPI) linkage. mRNA *in situ* hybridization (ISH) localized PSCA expression in normal prostate to the basal cell epithelium, the putative stem cell compartment of prostatic epithelium, suggesting that PSCA may be a marker of prostate stem/progenitor cells.

In order to examine the status of PSCA protein and mRNA expression in human Pca and validate it as a potential diagnostic and therapeutic target for Pca, we used immunohistochemistry (IHC) and *in situ* hybridization (ISH) simultaneously, and conducted PSCA protein and mRNA expression analyses in paraffin-embedded tissue specimens of benign prostatic hyperplasia (BPH, n = 20), prostate intraepithelial neoplasm (PIN, n = 20) and prostate cancer (Pca, n = 48). Furthermore, we evaluated the possible correlation of PSCA expression level with Pca tumorigenesis, grade, stage and progression to androgen-independence.

Materials and methods

Tissue samples

All of the clinical tissue specimens studied herein were obtained from 80 patients of 57–84 years old by prostatectomy, transurethral resection of prostate (TURP) or biopsies. The patients were classified as 20 cases of BPH, 20 cases of PIN, 40 cases of primary Pca, including 9 patients

with recurrent Pca and a history of androgen ablation therapy (orchiectomy and/or hormonal therapy), who were referred to as androgen-independent prostate cancers. Eight specimens were harvested from these androgen-independent Pca patients prior to androgen ablation treatment. Each tissue sample was cut into two parts, one was fixed in 10% formalin for IHC and the other treated with 4% paraformaldehyde/0.1 M PBS PH 7.4 in 0.1% DEPC for 1 h for ISH analysis, and then embedded in paraffin. All paraffin blocks examined were then cut into 5 μ m sections and mounted on the glass slides specific for IHC and ISH respectively in the usual fashion. H&E-stained section of each Pca was evaluated and assigned a Gleason score by the experienced urological pathologist at our institution based on the criteria of Gleason score [2]. The Gleason sums are summarized in Table 1. Clinical staging was performed according to Jewett-whitmore-prout staging system, as shown in Table 2. In the category of PIN, we graded the specimens into two groups, i.e. low grade PIN (grade I – II) and high grade PIN (HGPIN, grade III) on the basis of literatures [3,4].

Immunohistochemical (IHC) analysis

Briefly, tissue sections were deparaffinized, dehydrated, and subjected to microwaving in 10 mmol/L citrate buffer, PH 6.0 (Boshide, Wuhan, China) in a 900 W oven for 5 min to induce epitope retrieval. Slides were allowed to cool at room temperature for 30 min. A primary mouse antibody specific to human PSCA (Boshide, Wuhan, China) with a 1:100 dilution was applied to incubate with the slides at room temperature for 2 h. Labeling was detected by sequentially adding biotinylated secondary antibodies and streptavidin-peroxidase, and localized using 3,3'-diaminobenzidine reaction. Sections were then counterstained with hematoxylin. Substitution of the primary antibody with phosphate-buffered-saline (PBS) served as a negative-staining control.

mRNA *in situ* hybridization (ISH)

Five- μ m-thick tissue sections were deparaffinized and dehydrated, then digested in pepsin solution (4 mg/ml in 3% citric acid) for 20 min at 37.5°C, and further processed for ISH. Digoxigenin-labeled sense and antisense human PSCA RNA probes (obtained from Boshide, Wuhan, China) were hybridized to the sections at 48°C overnight. The posthybridization wash with a high stringency was performed sequentially at 37°C in 2 \times standard saline citrate (SSC) for 10 min, in 0.5 \times SSC for 15 min and in 0.2 \times SSC for 30 min. The slides were then incubated to biotinylated mouse anti-digoxigenin antibody at 37.5°C for 1 h followed by washing in 1 \times PBS for 20 min at room temperature, and then to streptavidin-peroxidase at 37.5°C for 20 min followed by washing in 1 \times PBS for 15 min at room temperature. Subsequently, the slides were developed with diaminobenzidine and then coun-

Table 1: Correlation of PSCA expression with Gleason score

Gleason score	Intensity × frequency	
	0-6 (%)	9 (%)
2-4	5 (83)	1 (17)
5-7	19 (79)	5 (21)
8-10	5 (28)	13 (72)

Table 2: Correlation of PSCA expression with clinical stage

Tumor stage	Intensity × frequency	
	0-6 (%)	9 (%)
≤B	27 (67.5)	13 (32.5)
≥C	2 (25)	6 (75)

terstained with hematoxylin to localize the hybridization signals. Sections hybridized with the sense control probes routinely did not show any specific hybridization signal above background. All slides were hybridized with PBS to substitute for the probes as a negative control.

Scoring methods

To determine the correlation between the results of PSCA immunostaining and mRNA *in situ* hybridization, the same scoring manners are taken in the present study for PSCA protein staining by IHC and PSCA mRNA staining by ISH. Each slide was read and scored by two independently experienced urological pathologists using Olympus BX-41 light microscopes. The evaluation was done in a blinded fashion. For each section, five areas of similar grade were analyzed semiquantitatively for the fraction of cells staining. Fifty percent of specimens were randomly chosen and rescored to determine the degree of interobserver and intraobserver concordance. There was greater than 95% intra- and interobserver agreement.

The intensity of PSCA expression evaluated microscopically was graded on a scale of 0 to 3+ with 3 being the highest expression observed (0, no staining; 1+, mildly intense; 2+, moderately intense; 3+, severely intense). The staining density was quantified as the percentage of cells staining positive for PSCA with the primary antibody or hybridization probe, as follows: 0 = no staining; 1 = positive staining in <25% of the sample; 2 = positive staining in 25%-50% of the sample; 3 = positive staining in >50%

of the sample. Intensity score (0 to 3+) was multiplied by the density score (0-3) to give an overall score of 0-9 [1,5]. In this way, we were able to differentiate specimens that may have had focal areas of increased staining from those that had diffuse areas of increased staining [6]. The overall score for each specimen was then categorically assigned to one of the following groups: 0 score, negative expression; 1-2 scores, weak expression; 3-6 scores, moderate expression; 9 score, strong expression.

Statistical analysis

Intensity and density of PSCA protein and mRNA expression in BPH, PIN and Pca tissues were compared using the Chi-square and Student's *t*-test. Univariate associations between PSCA expression and Gleason score, clinical stage and progression to androgen-independence were calculated using Fisher's Exact Test. For all analyses, $p < 0.05$ was considered statistically significant.

Results

PSCA expression in BPH

In general, PSCA protein and mRNA were expressed weakly in individual samples of BPH. Some areas of prostate expressed weak levels (composite score 1-2), whereas other areas were completely negative (composite score 0). Four cases (20%) of BPH had moderate expression of PSCA protein and mRNA (composite score 4-6) by IHC and ISH. In 2/20 (10%) BPH specimens, PSCA mRNA expression was moderate (composite score 3-6), but PSCA protein expression was weak (composite score

2) in one and negative (composite score 0) in the other. PSCA expression was localized to the basal and secretory epithelial cells, and prostatic stroma was almost negative staining for PSCA protein and mRNA in all cases examined.

PSCA expression in PIN

In this study, we detected weak or negative expression of PSCA protein and mRNA (≤ 2 scores) in 7 of 9 (77.8%) low grade PIN and in 2 of 11 (18.2%) HGPIN, and moderate expression (3–6 scores) in the rest 2 low grade PIN and 5 of 11 (45.5%) HGPIN. One HGPIN with moderate PSCA mRNA expression (6 score) was found weak staining for PSCA protein (2 score) by IHC. Strong PSCA protein and mRNA expression (9 score) were detected in the remaining 3 of 11 (27.3%) HGPIN. There was a statistically significant difference of PSCA protein and mRNA expression levels observed between HGPIN and BPH ($p < 0.05$), but no statistical difference reached between low grade PIN and BPH ($p > 0.05$).

PSCA expression in Pca

In order to determine if PSCA protein and mRNA can be detected in prostate cancers and if PSCA expression levels are increased in malignant compared with benign glands, Forty-eight paraffin-embedded Pca specimens were analysed by IHC and ISH. It was shown that 19 of 48 (39.6%) Pca samples stained very strongly for PSCA protein and mRNA with a score of 9 and another 21 (43.8%) specimens displayed moderate staining with scores of 4–6 (Figure 1). In addition, 4 specimens with moderate to strong PSCA mRNA expression (scores of 4–9) had weak protein staining (a score of 2) by IHC analyses. Overall, Pca expressed a significantly higher level of PSCA protein and mRNA than any other specimen category in this study ($p < 0.05$, compared with BPH and PIN respectively). The result demonstrates that PSCA protein and mRNA are overexpressed by a majority of human Pca.

Correlation of PSCA expression with Gleason score in Pca

Using the semi-quantitative scoring method as described in Materials and Methods, we compared the expression level of PSCA protein and mRNA with Gleason grade of Pca, as shown in Table 1. Prostate adenocarcinomas were graded by Gleason score as 2–4 scores = well-differentiation, 5–7 scores = moderate-differentiation and 8–10 scores = poor-differentiation [7]. Seventy-two percent of Gleason scores 8–10 prostate cancers had very strong staining of PSCA compared to 21% with Gleason scores 5–7 and 17% with 2–4 respectively, demonstrating that poorly differentiated Pca had significantly stronger expression of PSCA protein and mRNA than moderately and well differentiated tumors ($p < 0.05$). As depicted in Figure 1, IHC and ISH analyses showed that PSCA protein and mRNA expression in several cases of poorly differen-

tiated Pca were particularly prominent, with more intense and uniform staining. The results indicate that PSCA expression increases significantly with higher tumor grade in human Pca.

Correlation of PSCA expression with clinical stage in Pca

With regards to PSCA expression in every stage of Pca, we showed the results in Table 2. Seventy-five percent of locally advanced and node positive cancers (i.e. C-D stages) expressed statistically high levels of PSCA versus 32.5% that were organ confined (i.e. A-B stages) ($p < 0.05$). The data demonstrate that PSCA expression increases significantly with advanced tumor stage in human Pca.

Correlation of PSCA expression with androgen-independent progression of Pca

All 9 specimens of androgen-independent prostate cancers stained positive for PSCA protein and mRNA. Eight specimens were obtained from patients managed prior to androgen ablation therapy. Seven of eight (87.5%) of these androgen-independent prostate cancers were in the strongest staining category (score = 9), compared with three out of eight (37.5%) of patients with androgen-dependent cancers ($p < 0.05$). The results demonstrate that PSCA expression increases significantly with progression to androgen-independence of human Pca.

It is evident from the results above that within a majority of human prostate cancers the level of PSCA protein and mRNA expression correlates significantly with increasing grade, worsening stage and progression to androgen-independence.

Correlation of PSCA immunostaining and mRNA in situ hybridization

In all 88 specimens surveyed herein, we compared the results of PSCA IHC staining with mRNA ISH analysis. Positive staining areas and its intensity and density scores evaluated by IHC were identical to those seen by ISH in 79 of 88 (89.8%) specimens (18/20 BPH, 19/20 PIN and 42/48 Pca respectively). Importantly, 27/27 samples with PSCA mRNA composite scores of 0–2, 32/36 samples with scores of 3–6 and 22/24 samples with a score of 9 also had PSCA protein expression scores of 0–2, 3–6 and 9 respectively. However, in 5 samples with PSCA mRNA overall scores of 3–6 and in 2 with scores of 9 there were less or negative PSCA protein expression (i.e. scores of 0–4), suggesting that this may reflect posttranscriptional modification of PSCA or that the epitopes recognized by PSCA mAb may be obscured in some cancers. The data demonstrate that the results of PSCA immunostaining were consistent with those of mRNA ISH analysis, showing a high degree of correlation between PSCA protein and mRNA expression.

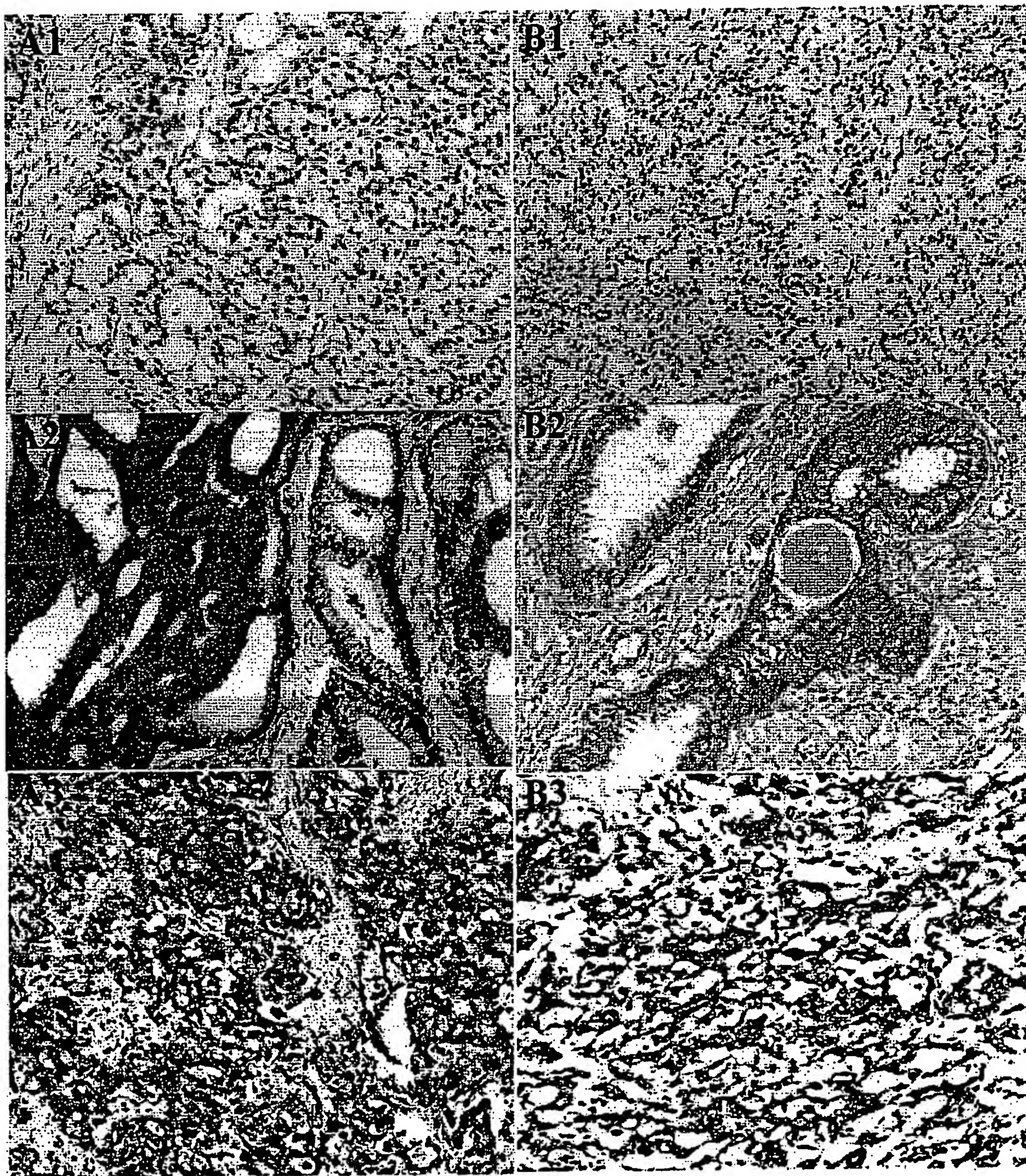


Figure 1

Representatives of PSCA IHC and ISH staining in Pca (A. IHC staining, B. ISH staining, $\times 200$ magnification). A₁, B₁: negative control of IHC and ISH. PBS replacing the primary antibody (A₁) and hybridization with a sense PSCA probe (B₁) showed no background staining. A₂, B₂: a moderately differentiated Pca (Gleason score = 3+3 = 6) with moderate staining (composite score = 6) in all malignant cells; A₂: IHC shows not only cell surface but also apparent cytoplasmic staining of PSCA protein. A₃, B₃: a poorly differentiated Pca (Gleason score = 4+4 = 8) with very strong staining (composite score = 9) in all malignant cells.

Discussion

PSCA is homologous to a group of cell surface proteins that mark the earliest phase of hematopoietic development. PSCA mRNA expression is prostate-specific in normal male tissues and is highly up-regulated in both androgen-dependent and-independent Pca xenografts (LAPC-4 tumors). We hypothesize that PSCA may play a role in Pca tumorigenesis and progression, and may serve as a target for Pca diagnosis and treatment. In this study, IHC and ISH showed that in general there were weak or absent PSCA protein and mRNA expression in BPH and low grade PIN tissues. However, PSCA protein and mRNA are widely expressed in HGPIN, the putative precursor of invasive Pca, suggesting that up-regulation of PSCA is an early event in prostate carcinogenesis. Recently, Reiter RE et al [1], using ISH analysis, reported that 97 of 118 (82%) HGPIN specimens stained strongly positive for PSCA mRNA. A very similar finding was seen on mouse PSCA (mPSCA) expression in mouse HGPIN tissues by Tran C. P et al [8]. These data suggest that PSCA may be a new marker associated with transformation of prostate cells and tumorigenesis.

We observed that PSCA protein and mRNA are highly expressed in a large percentage of human prostate cancers, including advanced, poorly differentiated, androgen-independent and metastatic cases. Fluorescence-activated cell sorting and confocal/ immunofluorescent studies demonstrated cell surface expression of PSCA protein in Pca cells [9]. Our IHC expression analysis of PSCA shows not only cell surface but also apparent cytoplasmic staining of PSCA protein in Pca specimens (Figure 1). One possible explanation for this is that anti-PSCA antibody can recognize PSCA peptide precursors that reside in the cytoplasm. Also, it is possible that the positive staining that appears in the cytoplasm is actually from the overlying cell membrane [5]. These data seem to indicate that PSCA is a novel cell surface marker for human Pca.

Our results show that elevated level of PSCA expression correlates with high grade (i.e. poor differentiation), increased tumor stage and progression to androgen-independence of Pca. These findings support the original IHC analyses by Gu Z et al [9], who reported that PSCA protein expressed in 94% of primary Pca and the intensity of PSCA protein expression increased with tumor grade, stage and progression to androgen-independence. Our results also collaborate the recent work of Han KR et al [10], in which the significant association between high PSCA expression and adverse prognostic features such as high Gleason score, seminal vesicle invasion and capsular involvement in Pca was found. It is suggested that PSCA overexpression may be an adverse predictor for recurrence, clinical progression or survival of Pca. Hara H et al [11] used RT-PCR detection of PSA, PSMA and PSCA in 1

ml of peripheral blood to evaluate Pca patients with poor prognosis. The results showed that among 58 Pca patients, each PCR indicated the prognostic value in the hierarchy of PSCA>PSA>PSMA RT-PCR, and extraprostatic cases with positive PSCA PCR indicated lower disease-progression-free survival than those with negative PSCA PCR, demonstrating that PSCA can be used as a prognostic factor. Dubey P et al [12] reported that elevated numbers of PSCA + cells correlate positively with the onset and development of prostate carcinoma over a long time span in the prostates of the TRAMP and PTEN +/- models compared with its normal prostates. Taken together with our present findings, in which PSCA is overexpressed from HGPIN to almost frank carcinoma, it is reasonable and possible to use increased PSCA expression level or increased numbers of PSCA-positive cells in the prostate samples as a prognostic marker to predict the potential onset of this cancer. These data raise the possibility that PSCA may have diagnostic utility or clinical prognostic value in human Pca.

The cause of PSCA overexpression in Pca is not known. One possible mechanism is that it may result from PSCA gene amplification. In humans, PSCA is located on chromosome 8q24.2 [1], which is often amplified in metastatic and recurrent Pca and considered to indicate a poor prognosis [13-15]. Interestingly, PSCA is in close proximity to the c-myc oncogene, which is amplified in >20% of recurrent and metastatic prostate cancers [16,17]. Reiter RE et al [18] reported that PSCA and MYC gene copy numbers were co-amplified in 25% of tumors (five out of twenty), demonstrating that PSCA overexpression is associated with PSCA and MYC coamplification in Pca. Gu Z et al [9] recently reported that in 102 specimens available to compare the results of PSCA immunostaining with their previous mRNA ISH analysis, 92 (90.2%) had identically positive areas of PSCA protein and mRNA expression. Taken together with our findings, in which we detected moderate to strong expression of PSCA protein and mRNA in 34 of 40 (85%) Pca specimens examined simultaneously by IHC and ISH analyses, it is demonstrated that PSCA protein and mRNA overexpressed in human Pca, and that the increased protein level of PSCA was resulted from the upregulated transcription of its mRNA.

At present, the regulation mechanisms of human PSCA expression and its biological function are yet to be elucidated. PSCA expression may be regulated by multiple factors [18]. Watabe T et al [19] reported that transcriptional control is a major component regulating PSCA expression levels. In addition, induction of PSCA expression may be regulated or mediated through cell-cell contact and protein kinase C (PKC) [20]. Homologues of PSCA have diverse activities, and have themselves been involved in

carcinogenesis. Signalling through SCA-2 has been demonstrated to prevent apoptosis in immature thymocytes [21]. Thy-1 is involved in T cell activation and transduces signals through src-like tyrosine kinases [22]. Ly-6 genes have been implicated both in tumorigenesis and in cell-cell adhesion [23-25]. Cell-cell or cell-matrix interaction is critical for local tumor growth and spread to distal sites. From its restricted expression in basal cells of normal prostate and its homology to SCA-2, PSCA may play a role in stem/progenitor cell function, such as self-renewal (i.e. anti-apoptosis) and/or proliferation [1]. Taken together with the results in the present study, we speculate that PSCA may play a role in tumorigenesis and clinical progression of Pca through affecting cell transformation and proliferation. From our results, it is also suggested that PSCA as a new cell surface antigen may have a number of potential uses in the diagnosis, therapy and clinical prognosis of human Pca. PSCA overexpression in prostate biopsies could be used to identify patients at high risk to develop recurrent or metastatic disease, and to discriminate cancers from normal glands in prostatectomy samples. Similarly, the detection of PSCA-overexpressing cells in bone marrow or peripheral blood may identify and predict metastatic progression better than current assays, which identify only PSA-positive or PSMA-positive prostate cells.

In summary, we have shown in this study that PSCA protein and mRNA are maintained in expression from HGPIN through all stages of Pca in a majority of cases, which may be associated with prostate carcinogenesis and correlate positively with high tumor grade (poor cell differentiation), advanced stage and androgen-independent progression. PSCA protein overexpression is due to the upregulation of its mRNA transcription. The results suggest that PSCA may be a promising molecular marker for the clinical prognosis of human Pca and a valuable target for diagnosis and therapy of this tumor.

Competing interests

None declared.

References

- Reiter RE, Gu Z, Watabe T, Thomas G, Szigeti K, David E, Wahl M, Nisitani S, Yamashiro J, Le Beau MM, Loda M, Witte ON: Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. *Proc Natl Acad Sci USA* 1998, 95:1735-1740.
- Gleason DF: Histologic grading and clinical staging of prostatic carcinoma. In: *Urologic Pathology: The Prostate* Edited by: Tannebaum M. Philadelphia, Lea & Febiger; 1977:171-197.
- Brawer MK: Prostatic intraepithelial neoplasia: a premalignant lesion. *Hum Pathol* 1992, 23:242-248.
- Amin MB, Ro JY, Ayala AC: Prostatic intraepithelial neoplasia: relationship to adenocarcinoma of prostate. *Pathol Annu* 1994, 29:1-30.
- Amara N, Palapattu GS, Schrage M, Gu Z, Thomas GV, Dorey F, Said J, Reiter RE: Prostate stem cell antigen is overexpressed in human transitional cell carcinoma. *Cancer Res* 2001, 61:4660-4665.
- Hanas JS, Lerner MR, Lightfoot SA, Raczowski C, Kastens DJ, Brackett DJ, Posder RG: Expression of the cyclin-dependent kinase inhibitor p21 (WAF1/CIP1) and p53 tumor suppressor in dysplastic progression and adenocarcinoma in Barrett esophagus. *Cancer (Phila)* 1999, 86:756-763.
- Egevad L, Gramfors T, Karlberg L: Prognostic value of the Gleason score in prostate cancer. *BJU Int* 2002, 89:538-542.
- Tran CP, Lin C, Yamashiro J, Reiter RE: Prostate stem cell antigen is a marker of late intermediate prostate epithelial cells. *Mol Cancer Res* 2002, 1:113-121.
- Gu Z, Thomas G, Yamashiro J, Shintaku IP, Dorey F, Raitano A, Witte ON, Said JW, Loda M, Reiter RE: Prostate stem cell antigen (PSCA) expression increases with high Gleason score, advanced stage and bone metastasis in prostate cancer. *Oncogene* 2000, 19:1288-1296.
- Han KR, Seligson DB, Liu X, Horvath S, Shintaku PI, Thomas GV, Said JW, Reiter RE: Prostate stem cell antigen expression is associated with gleason score, seminal vesicle invasion and capsular invasion in prostate cancer. *J Urol* 2004, 171:1117-1121.
- Hara H, Kasahara T, Kawasaki T, Bilim V, Obara K, Takahashi K, Tomita Y: Reverse Transcription-Polymerase Chain Reaction Detection of Prostate-specific Antigen, Prostate-specific Membrane Antigen, and Prostate Stem Cell Antigen in One Milliliter of Peripheral Blood. *Clin Cancer Res* 2002, 8:1794-1799.
- Dubey P, Wu H, Reiter RE, Witte ON: Alternative pathways to prostate carcinoma activate prostate stem cell antigen expression. *Cancer Res* 2001, 61:3256-3261.
- Visakorpi T, Kallioniemi AH, Syvanen AC, Hyytiäinen ER, Karhu R, Tammela T, Isola JJ, Kallioniemi OP: Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. *Cancer Res* 1995, 55:342-347.
- Sato K, Qian J, Slezak JM, Lieber MM, Bostwick DG, Bergstrahl EJ, Jenkins RB: Clinical significance of alterations of chromosome 8 in high-grade, advanced, nonmetastatic prostate carcinoma. *J Natl Cancer Inst* 1999, 91:1574-1580.
- Van Den Berg C, Guan XY, Von Hoff D, Jenkins R, Bitner C, Griffin C, Kallioniemi O, Visakorpi T, McGill J, Herath J, Epstein J, Sarosdy M, Meltzer P, Trent J: DNA sequence amplification in human prostate cancer identified by chromosome microdissection: potential prognostic implications. *Clin Cancer Res* 1995, 1:11-18.
- Jenkins RB, Qian J, Lieber MM, Bostwick DG: Detection of c-myc oncogene amplification and chromosomal anomalies in metastatic prostatic carcinoma by fluorescence in situ hybridization. *Cancer Res* 1997, 57:524-531.
- Nupponen NN, Kakkola L, Koivisto P, Visakorpi T: Genetic alterations in hormone-refractory recurrent prostate carcinomas. *Am J Pathol* 1998, 153:141-148.
- Reiter RE, Sato I, Thomas G, Qian J, Gu Z, Watabe T, Loda M, Jenkins RB: Coamplification of prostate stem cell antigen (PSCA) and MYC in locally advanced prostate cancer. *Genes Chromosomes Cancer* 2000, 27:95-103.
- Watabe T, Lin M, Donjacour AA, Cunha GR, Witte ON, Reiter RE: Growth, regeneration, and tumorigenesis of the prostate activates the PSCA promoter. *Proc Natl Acad Sci USA* 2002, 99:401-406.
- Bahrenberg G, Brauers A, Joost HG, Jakse G: PSCA expression is regulated by phorbol ester and cell adhesion in the bladder carcinoma cell line RT112. *Cancer Lett* 2001, 168:37-43.
- Noda S, Kosugi A, Saitoh S, Narumiya S, Hamaoka T: Protection from anti-TCR/CD3-induced apoptosis in immature thymocytes by a signal through thymic shared antigen-1/stem cell antigen-2. *J Exp Med* 1996, 183:2355-2360.
- Thomas PM, Samelson LE: The glycosylphosphatidylinositol-anchored Thy-1 molecule interacts with the p60fyn protein tyrosine kinase in T cells. *J Biol Chem* 1992, 267:12317-12322.
- Bamezai A, Rock KL: Overexpressed Ly-6A.2 mediated cell-cell adhesion by binding a ligand expressed on lymphoid cells. *Proc Natl Acad Sci USA* 1995, 92:4294-4298.
- Katz BZ, Eshel R, Sagi-Assif O, Witz IP: An association between high Ly-6A/E expression on tumor cells and a highly malignant phenotype. *Int J Cancer* 1994, 59:684-691.
- Brakenhoff RH, Gerretsen M, Knippels EM, van Dijk M, van Essen H, Weghuis DO, Sinke RJ, Snow GB, van Dongen GA: The human E48 antigen, highly homologous to the murine Ly-6 antigen ThB, is a GPI-anchored molecule apparently involved in keratinocyte cell-cell adhesion. *J Cell Biol* 1995, 129:1677-1689.

Review

Translation Initiation in Cancer: A Novel Target for Therapy¹

Funda Meric² and Kelly K. Hunt

Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

Translation initiation is regulated in response to nutrient availability and mitogenic stimulation and is coupled with cell cycle progression and cell growth. Several alterations in translational control occur in cancer. Variant mRNA sequences can alter the translational efficiency of individual mRNA molecules, which in turn play a role in cancer biology. Changes in the expression or availability of components of the translational machinery and in the activation of translation through signal transduction pathways can lead to more global changes, such as an increase in the overall rate of protein synthesis and translational activation of the mRNA molecules involved in cell growth and proliferation. We review the basic principles of translational control, the alterations encountered in cancer, and selected therapies targeting translation initiation to help elucidate new therapeutic avenues.

Introduction

The fundamental principle of molecular therapeutics in cancer is to exploit the differences in gene expression between cancer cells and normal cells. With the advent of cDNA array technology, most efforts have concentrated on identifying differences in gene expression at the level of mRNA, which can be attributable either to DNA amplification or to differences in transcription. Gene expression is quite complicated, however, and is also regulated at the level of mRNA stability, mRNA translation, and protein stability.

The power of translational regulation has been best recognized among developmental biologists, because transcription does not occur in early embryogenesis in eukaryotes. For example, in *Xenopus*, the period of transcriptional quiescence continues until the embryo reaches midblastula transition, the 4000-cell stage. Therefore, all necessary mRNA molecules are transcribed during oogenesis and stockpiled in a translationally inactive, masked form. The mRNA are translationally activated at appropriate times during oocyte maturation, fertilization, and

early embryogenesis and thus, are under strict translational control.

Translation has an established role in cell growth. Basically, an increase in protein synthesis occurs as a consequence of mitogenesis. Until recently, however, little was known about the alterations in mRNA translation in cancer, and much is yet to be discovered about their role in the development and progression of cancer. Here we review the basic principles of translational control, the alterations encountered in cancer, and selected therapies targeting translation initiation to elucidate potential new therapeutic avenues.

Basic Principles of Translational Control

Mechanism of Translation Initiation

Translation initiation is the main step in translational regulation. Translation initiation is a complex process in which the initiator tRNA and the 40S and 60S ribosomal subunits are recruited to the 5' end of a mRNA molecule and assembled by eukaryotic translation initiation factors into an 80S ribosome at the start codon of the mRNA (Fig. 1). The 5' end of eukaryotic mRNA is capped, i.e., contains the cap structure m⁷GpppN (7-methylguanosine-triphospho-5'-ribonucleoside). Most translation in eukaryotes occurs in a cap-dependent fashion, i.e., the cap is specifically recognized by the eIF4E,³ which binds the 5' cap. The eIF4F translation initiation complex is then formed by the assembly of eIF4E, the RNA helicase eIF4A, and eIF4G, a scaffolding protein that mediates the binding of the 40S ribosomal subunit to the mRNA molecule through interaction with the eIF3 protein present on the 40S ribosome. eIF4A and eIF4B participate in melting the secondary structure of the 5' UTR of the mRNA. The 43S initiation complex (40S/eIF2/Met-tRNA/GTP complex) scans the mRNA in a 5'→3' direction until it encounters an AUG start codon. This start codon is then base-paired to the anticodon of initiator tRNA, forming the 48S initiation complex. The initiation factors are then displaced from the 48S complex, and the 60S ribosome joins to form the 80S ribosome.

Unlike most eukaryotic translation, translation initiation of certain mRNAs, such as the picornavirus RNA, is cap independent and occurs by internal ribosome entry. This mechanism does not require eIF4E. Either the 43S complex can bind the initiation codon directly through interaction with the IRES in the 5' UTR such as in the encephalomyocarditis virus, or it can

Received 5/16/02; revised 7/12/02; accepted 7/22/02.

¹ F. M. is supported by The University of Texas M. D. Anderson Cancer Center Physician-Scientist Program and by NIH Grant 1K08-CA 91895-01. K. K. H. is supported by Department of Defense Award DAMD-17-97-1-7162.

² To whom requests for reprints should be addressed, at Department of Surgical Oncology, Box 444, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 745-4453; Fax: (713) 745-4926; E-mail: fmeric@mdanderson.org.

³ The abbreviations used are: eIF4E, eukaryotic initiation factor 4E; UTR, untranslated region; IRES, internal ribosome entry site; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; S6K, ribosomal p70 S6 kinase; mTOR, mammalian target of rapamycin; ATM, ataxia telangiectasia mutated; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog deleted from chromosome 10; PP2A, protein phosphatase 2A; TGF- β 3, transforming growth factor- β 3; PAP, poly(A) polymerase; EPA, eicosapentaenoic acid; mda-7, melanoma differentiation-associated gene 7.

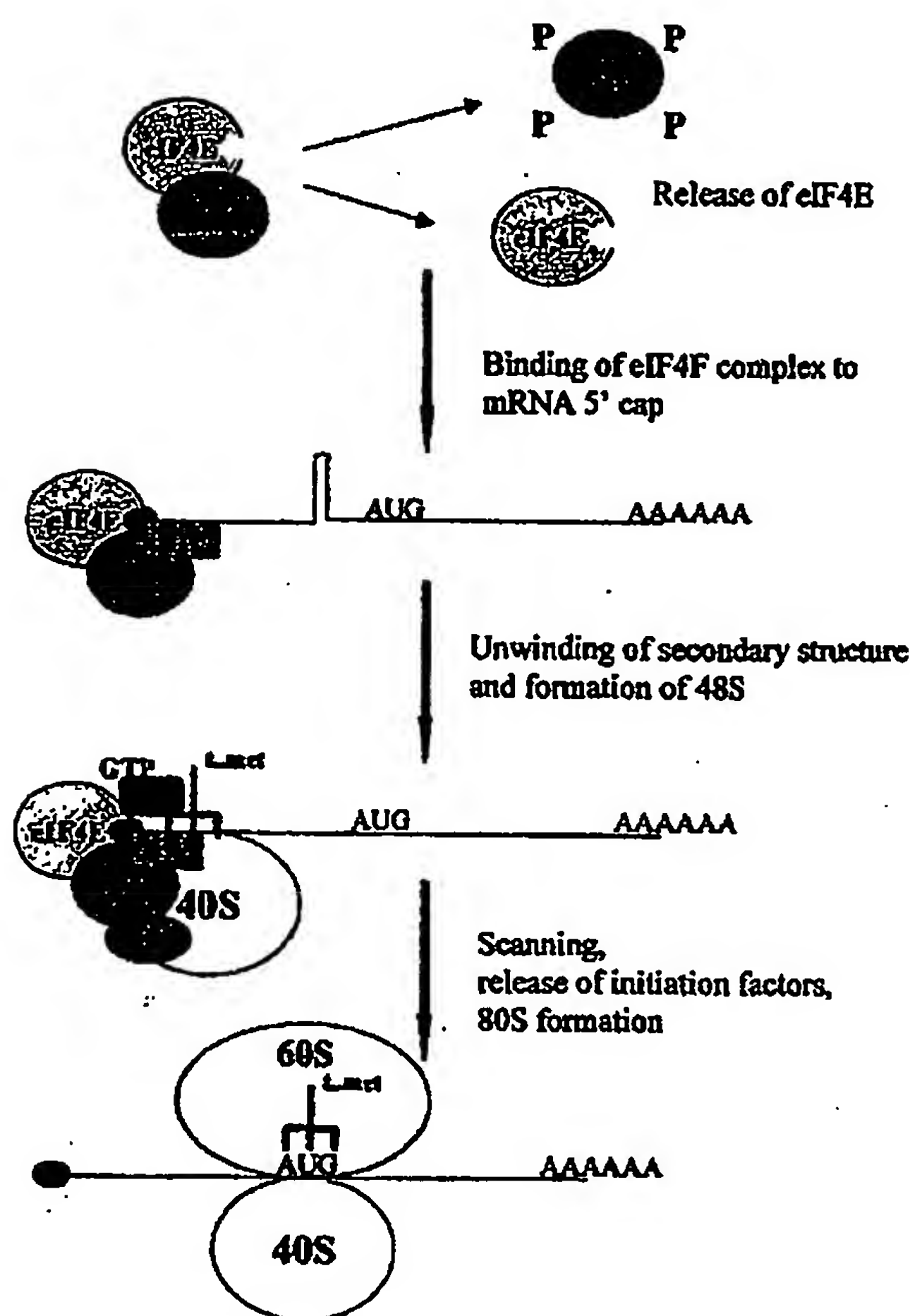


Fig. 1. Translation initiation in eukaryotes. The 4E-BPs are hyperphosphorylated to release eIF4E so that it can interact with the 5' cap, and the eIF4F initiation complex is assembled. The interaction of poly(A) binding protein with the initiation complex and circularization of the mRNA is not depicted in the diagram. The secondary structure of the 5' UTR is melted, the 40S ribosomal subunit is bound to eIF3, and the ternary complex consisting of eIF2, GTP, and the Met-tRNA are recruited to the mRNA. The ribosome scans the mRNA in a 5'→3' direction until an AUG start codon is found in the appropriate sequence context. The initiation factors are released, and the large ribosomal subunit is recruited.

Initially attach to the IRES and then reach the initiation codon by scanning or transfer, as is the case with the poliovirus (1).

Regulation of Translation Initiation

Translation initiation can be regulated by alterations in the expression or phosphorylation status of the various factors involved. Key components in translational regulation that may provide potential therapeutic targets follow.

eIF4E. eIF4E plays a central role in translation regulation. It is the least abundant of the initiation factors and is considered the rate-limiting component for initiation of cap-dependent translation. eIF4E may also be involved in mRNA splicing, mRNA 3' processing, and mRNA nucleocytoplasmic transport (2). eIF4E expression can be increased at the transcriptional level in response to serum or growth factors (3). eIF4E overexpression may cause preferential translation of mRNAs containing excessive secondary structure in their 5' UTR that are normally discriminated against by the trans-

lational machinery and thus are inefficiently translated (4–7). As examples of this, overexpression of eIF4E promotes increased translation of vascular endothelial growth factor, fibroblast growth factor-2, and cyclin D1 (2, 8, 9).

Another mechanism of control is the regulation of eIF4E phosphorylation. eIF4E phosphorylation is mediated by the mitogen-activated protein kinase-interacting kinase 1, which is activated by the mitogen-activated pathway activating extracellular signal-related kinases and the stress-activated pathway acting through p38 mitogen-activated protein kinase (10–13). Several mitogens, such as serum, platelet-derived growth factor, epidermal growth factor, insulin, angiotensin II, src kinase overexpression, and ras overexpression, lead to eIF4E phosphorylation (14). The phosphorylation status of eIF4E is usually correlated with the translational rate and growth status of the cell; however, eIF4E phosphorylation has also been observed in response to some cellular stresses when translational rates actually decrease (15). Thus, further study is needed to understand the effects of eIF4E phosphorylation on eIF4E activity.

Another mechanism of regulation is the alteration of eIF4E availability by the binding of eIF4E to the eIF4E-binding proteins (4E-BP, also known as PHAS-I). 4E-BPs compete with eIF4G for a binding site in eIF4E. The binding of eIF4E to the best characterized eIF4E-binding protein, 4E-BP1, is regulated by 4E-BP1 phosphorylation. Hypophosphorylated 4E-BP1 binds to eIF4E, whereas 4E-BP1 hyperphosphorylation decreases this binding. Insulin, angiotensin, epidermal growth factor, platelet-derived growth factor, hepatocyte growth factor, nerve growth factor, insulin-like growth factors I and II, interleukin 3, granulocyte-macrophage colony-stimulating factor + steel factor, gastrin, and the adenovirus have all been reported to induce phosphorylation of 4E-BP1 and to decrease the ability of 4E-BP1 to bind eIF4E (15, 16). Conversely, deprivation of nutrients or growth factors results in 4E-BP1 dephosphorylation, an increase in eIF4E binding, and a decrease in cap-dependent translation.

p70 S6 Kinase. Phosphorylation of ribosomal 40S protein S6 by S6K is thought to play an important role in translational regulation. S6K $-/-$ mouse embryonic cells proliferate more slowly than do parental cells, demonstrating that S6K has a positive influence on cell proliferation (17). S6K regulates the translation of a group of mRNAs possessing a 5' terminal oligopyrimidine tract (5' TOP) found at the 5' UTR of ribosomal protein mRNAs and other mRNAs coding for components of the translational machinery. Phosphorylation of S6K is regulated in part based on the availability of nutrients (18, 19) and is stimulated by several growth factors, such as platelet-derived growth factor and insulin-like growth factor I (20).

eIF2 α Phosphorylation. The binding of the initiator tRNA to the small ribosomal unit is mediated by translation initiation factor eIF2. Phosphorylation of the α -subunit of eIF2 prevents formation of the eIF2/GTP/Met-tRNA complex and inhibits global protein synthesis (21, 22). eIF2 α is phosphorylated under a variety of conditions, such as viral infection, nutrient deprivation, heme deprivation, and apoptosis (22). eIF2 α is phosphorylated by heme-regulated inhibitor, nutrient-regulated protein kinase, and the IFN-induced, double-stranded RNA-activated protein kinase (PKR; Ref. 23).

The mTOR Signaling Pathway. The macrolide antibiotic rapamycin (Siralimus; Wyeth-Ayerst Research, Collegeville, PA) has been the subject of intensive study because it inhibits signal transduction pathways involved in T-cell activation. The rapamycin-sensitive component of these pathways is mTOR (also called FRAP or RAFT1). mTOR is the mammalian homologue of the yeast TOR proteins that regulate G₁ progression and translation in response to nutrient availability (24). mTOR is a serine-threonine kinase that modulates translation initiation by altering the phosphorylation status of 4E-BP1 and S6K (Fig. 2; Ref. 25).

4E-BP1 is phosphorylated on multiple residues. mTOR phosphorylates the Thr-37 and Thr-46 residues of 4E-BP1 *in vitro* (26); however, phosphorylation at these sites is not associated with a loss of eIF4E binding. Phosphorylation of Thr-37 and Thr-46 is required for subsequent phosphorylation at several COOH-terminal, serum-sensitive sites; a combination of these phosphorylation events appears to be needed to inhibit the binding of 4E-BP1 to eIF4E (25). The product of the ATM gene, p38/MSK1 pathway, and protein kinase C α also play a role in 4E-BP1 phosphorylation (27–29).

S6K and 4E-BP1 are also regulated, in part, by PI3K and its downstream protein kinase Akt. PTEN is a phosphatase that negatively regulates PI3K signaling. PTEN null cells have constitutively active Akt, with increased S6K activity and S6 phosphorylation (30). S6K activity is inhibited both by PI3K inhibitors wortmannin and LY294002 and by mTOR inhibitor rapamycin (24). Akt phosphorylates Ser-2448 in mTOR *in vitro*, and this site is phosphorylated upon Akt activation *in vivo* (31–33). Thus, mTOR is regulated by the PI3K/Akt pathway; however, this does not appear to be the only mode of regulation of mTOR activity. Whether the PI3K pathway also regulates S6K and 4E-BP1 phosphorylation independent of mTOR is controversial.

Interestingly, mTOR autophosphorylation is blocked by wortmannin but not by rapamycin (34). This seeming inconsistency suggests that mTOR-responsive regulation of 4E-BP1 and S6K activity occurs through a mechanism other than intrinsic mTOR kinase activity. An alternate pathway for 4E-BP1 and S6K phosphorylation by mTOR activity is by the inhibition of a phosphatase. Treatment with calyculin A, an inhibitor of phosphatases 1 and 2A, reduces rapamycin-induced dephosphorylation of 4E-BP1 and S6K by rapamycin (35). PP2A interacts with full-length S6K but not with a S6K mutant that is resistant to dephosphorylation resulting from rapamycin. mTOR phosphorylates PP2A *in vitro*; however, how this process alters PP2A activity is not known. These results are consistent with the model that phosphorylation of a phosphatase by mTOR prevents dephosphorylation of 4E-BP1 and S6K, and conversely, that nutrient deprivation and rapamycin block inhibition of the phosphatase by mTOR.

Polyadenylation. The poly(A) tail in eukaryotic mRNA is important in enhancing translation initiation and mRNA stability. Polyadenylation plays a key role in regulating gene expression during oogenesis and early embryogenesis. Some mRNA that are translationally inactive in the oocyte are polyadenylated concomitantly with translational activation in oocyte maturation, whereas other mRNAs that are translationally active during oogenesis are deadenylated and trans-

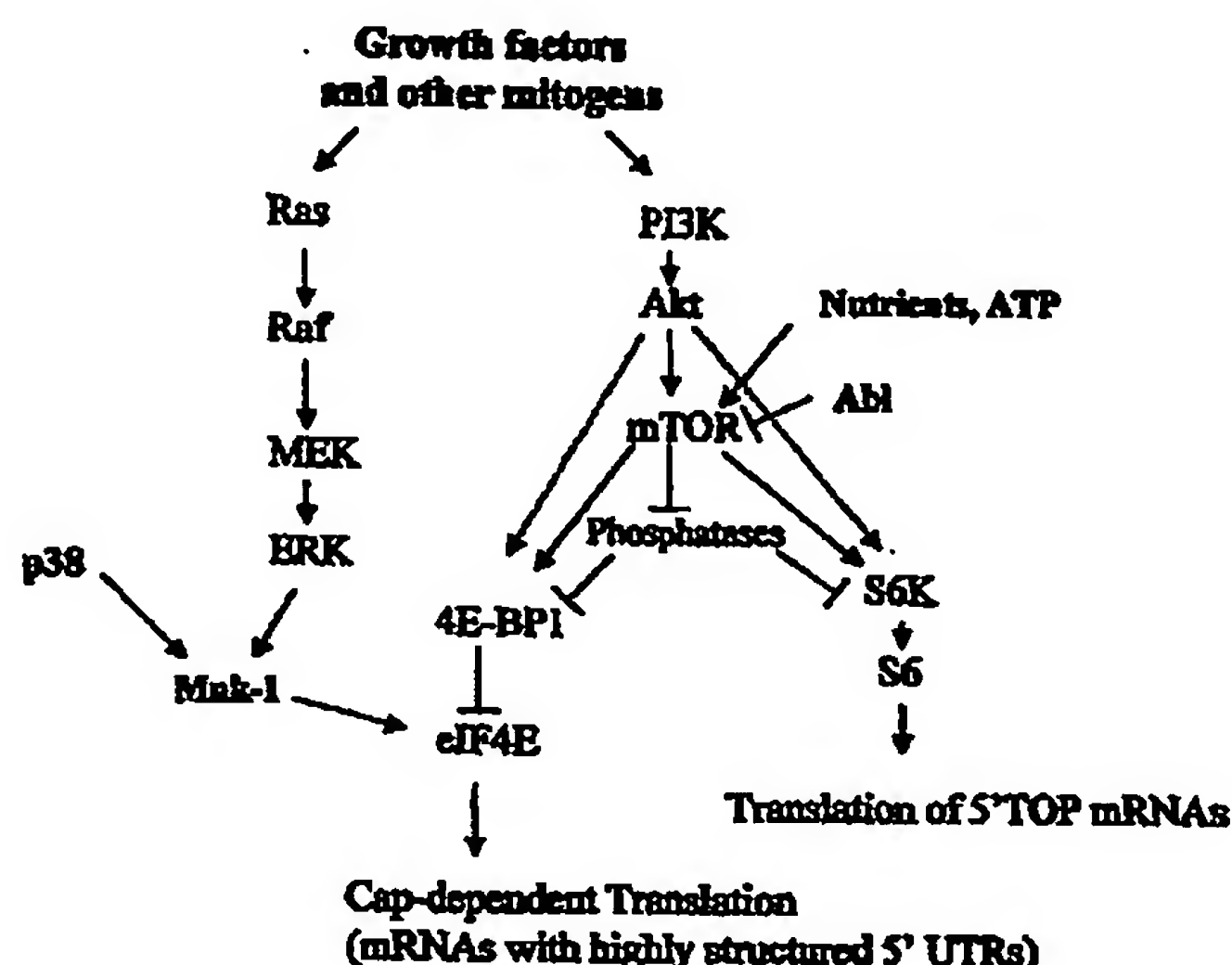


Fig. 2. Regulation of translation initiation by signal transduction pathways. Signaling via p38, extracellular signal-related kinase, PI3K, and mTOR can all activate translation initiation.

lationally silenced (36–38). Thus, control of poly(A) tail synthesis is an important regulatory step in gene expression. The 5' cap and poly(A) tail are thought to function synergistically to regulate mRNA translational efficiency (39, 40).

RNA Packaging. Most RNA-binding proteins are assembled on a transcript at the time of transcription, thus determining the translational fate of the transcript (41). A highly conserved family of Y-box proteins is found in cytoplasmic messenger ribonucleoprotein particles, where the proteins are thought to play a role in restricting the recruitment of mRNA to the translational machinery (41–43). The major mRNA-associated protein, YB-1, destabilizes the interaction of eIF4E and the 5' mRNA cap *in vitro*, and overexpression of YB-1 results in translational repression *in vivo* (44). Thus, alterations in RNA packaging can also play an important role in translational regulation.

Translation Alterations Encountered in Cancer

Three main alterations at the translational level occur in cancer: variations in mRNA sequences that increase or decrease translational efficiency, changes in the expression or availability of components of the translational machinery, and activation of translation through aberrantly activated signal transduction pathways. The first alteration affects the translation of an individual mRNA that may play a role in carcinogenesis. The second and third alterations can lead to more global changes, such as an increase in the overall rate of protein synthesis, and the translational activation of several mRNA species.

Variations in mRNA Sequence

Variations in mRNA sequence affect the translational efficiency of the transcript. A brief description of these variations and examples of each mechanism follow.

Mutations. Mutations in the mRNA sequence, especially in the 5' UTR, can alter its translational efficiency, as seen in the following examples.

c-myc. Saito *et al.* proposed that translation of full-length c-myc is repressed, whereas in several Burkitt lymphomas that have deletions of the mRNA 5' UTR, translation of c-myc is more efficient (45). More recently, it was reported that the 5' UTR of c-myc contains an IRES, and thus c-myc translation can be initiated by a cap-independent as well as a cap-dependent mechanism (46, 47). In patients with multiple myeloma, a C→T mutation in the c-myc IRES was identified (48) and found to cause an enhanced initiation of translation via internal ribosomal entry (49).

BRCA1. A somatic point mutation (117 G→C) in position -3 with respect to the start codon of the BRCA1 gene was identified in a highly aggressive sporadic breast cancer (50). Chimeric constructs consisting of the wild-type or mutated BRCA1 5' UTR and a downstream luciferase reporter demonstrated a decrease in the translational efficiency with the 5' UTR mutation.

Cyclin-dependent Kinase Inhibitor 2A. Some inherited melanoma kindreds have a G→T transversion at base -34 of cyclin-dependent kinase inhibitor-2A, which encodes a cyclin-dependent kinase 4/cyclin-dependent kinase 6 kinase inhibitor important in G₁ checkpoint regulation (51). This mutation gives rise to a novel AUG translation initiation codon, creating an upstream open reading frame that competes for scanning ribosomes and decreases translation from the wild-type AUG.

Alternate Splicing and Alternate Transcription Start Sites. Alterations in splicing and alternate transcription sites can lead to variations in 5' UTR sequence, length, and secondary structure, ultimately impacting translational efficiency.

ATM. The ATM gene has four noncoding exons in its 5' UTR that undergo extensive alternative splicing (52). The contents of 12 different 5' UTRs that show considerable diversity in length and sequence have been identified. These divergent 5' leader sequences play an important role in the translational regulation of the ATM gene.

mdm. In a subset of tumors, overexpression of the oncoprotein mdm2 results in enhanced translation of the mdm2 mRNA. Use of different promoters leads to two mdm2 transcripts that differ only in their 5' leaders (53). The longer 5' UTR contains two upstream open reading frames, and this mRNA is loaded with ribosomes inefficiently compared with the short 5' UTR.

BRCA1. In a normal mammary gland, BRCA1 mRNA is expressed with a shorter leader sequence (5' UTRa), whereas in sporadic breast cancer tissue, BRCA1 mRNA is expressed with a longer leader sequence (5' UTRb); the translational efficiency of transcripts containing 5' UTRb is 10 times lower than that of transcripts containing 5' UTRa (54).

TGF-β3. TGF-β3 mRNA includes a 1.1-kb 5' UTR, which exerts an inhibitory effect on translation. Many human breast cancer cell lines contain a novel TGF-β3 transcript with a 5' UTR that is 870 nucleotides shorter and has a 7-fold greater translational efficiency than the normal TGF-β3 mRNA (55).

Alternate Polyadenylation Sites. Multiple polyadenylation signals leading to the generation of several transcripts with differing 3' UTR have been described for several mRNA species, such as the RET proto-oncogene (56), ATM gene (52), tissue inhibitor of metalloproteinases-3 (57), RHOA

proto-oncogene (58), and calmodulin-I (59). Although the effect of these alternate 3' UTRs on translation is not yet known, they may be important in RNA-protein interactions that affect translational recruitment. The role of these alterations in cancer development and progression is unknown.

Alterations in the Components of the Translation Machinery

Alterations in the components of translation machinery can take many forms.

Overexpression of eIF4E. Overexpression of eIF4E causes malignant transformation in rodent cells (60) and the deregulation of HeLa cell growth (61). Polunovsky *et al.* (62) found that eIF4E overexpression substitutes for serum and individual growth factors in preserving viability of fibroblasts, which suggests that eIF4E can mediate both proliferative and survival signaling.

Elevated levels of eIF4E mRNA have been found in a broad spectrum of transformed cell lines (63). eIF4E levels are elevated in all ductal carcinoma *in situ* specimens and invasive ductal carcinomas, compared with benign breast specimens evaluated with Western blot analysis (64, 65). Preliminary studies suggest that this overexpression is attributable to gene amplification (66).

There are accumulating data suggesting that eIF4E overexpression can be valuable as a prognostic marker. eIF4E overexpression was found in a retrospective study to be a marker of poor prognosis in stages I to III breast carcinoma (67). Verification of the prognostic value of eIF4E in breast cancer is now under way in a prospective trial (67). However, in a different study, eIF4E expression was correlated with the aggressive behavior of non-Hodgkin's lymphomas (68). In a prospective analysis of patients with head and neck cancer, elevated levels of eIF4E in histologically tumor-free surgical margins predicted a significantly increased risk of local-regional recurrence (9). These results all suggest that eIF4E overexpression can be used to select patients who might benefit from more aggressive systemic therapy. Furthermore, the head and neck cancer data suggest that eIF4E overexpression is a field defect and can be used to guide local therapy.

Alterations in Other Initiation Factors. Alterations in a number of other initiation factors have been associated with cancer. Overproduction of eIF4G, similar to eIF4E, leads to malignant transformation *in vitro* (69). eIF-2α is found in increased levels in bronchioloalveolar carcinomas of the lung (3). Initiation factor eIF-4A1 is overexpressed in melanoma (70) and hepatocellular carcinoma (71). The p40 subunit of translation initiation factor 3 is amplified and overexpressed in breast and prostate cancer (72), and the eIF3-p110 subunit is overexpressed in testicular seminoma (73). The role that overexpression of these initiation factors plays on the development and progression of cancer, if any, is not known.

Overexpression of S6K. S6K is amplified and highly overexpressed in the MCF7 breast cancer cell line, compared with normal mammary epithelium (74). In a study by Barlund *et al.* (74), S6K was amplified in 59 of 668 primary breast tumors, and a statistically significant association was observed between amplification and poor prognosis.

Overexpression of PAP. PAP catalyzes 3' poly(A) synthesis. PAP is overexpressed in human cancer cells compared with normal and virally transformed cells (75). PAP enzymatic activity in breast tumors has been correlated with PAP protein levels (76) and, in mammary tumor cytosols, was found to be an independent factor for predicting survival (76). Little is known, however, about how PAP expression or activity affects the translational profile.

Alterations in RNA-binding Proteins. Even less is known about alterations in RNA packaging in cancer. Increased expression and nuclear localization of the RNA-binding protein YB-1 are indicators of a poor prognosis for breast cancer (77), non-small cell lung cancer (78), and ovarian cancer (79). However, this effect may be mediated at least in part at the level of transcription, because YB-1 increases chemoresistance by enhancing the transcription of a multidrug resistance gene (80).

Activation of Signal Transduction Pathways

Activation of signal transduction pathways by loss of tumor suppressor genes or overexpression of certain tyrosine kinases can contribute to the growth and aggressiveness of tumors. An important mutant in human cancers is the tumor suppressor gene *PTEN*, which leads to the activation of the PI3K/Akt pathway. Activation of PI3K and Akt induces the oncogenic transformation of chicken embryo fibroblasts. The transformed cells show constitutive phosphorylation of S6K and of 4E-BP1 (81). A mutant Akt that retains kinase activity but does not phosphorylate S6K or 4E-BP1 does not transform fibroblasts, which suggests a correlation between the oncogenicity of PI3K and Akt and the phosphorylation of S6K and 4E-BP1 (81).

Several tyrosine kinases such as platelet-derived growth factor, insulin-like growth factor, HER2/neu, and epidermal growth factor receptor are overexpressed in cancer. Because these kinases activate downstream signal transduction pathways known to alter translation initiation, activation of translation is likely to contribute to the growth and aggressiveness of these tumors. Furthermore, the mRNA for many of these kinases themselves are under translational control. For example, HER2/neu mRNA is translationally controlled both by a short upstream open reading frame that represses HER2/neu translation in a cell type-independent manner and by a distinct cell type-dependent mechanism that increases translational efficiency (82). HER2/neu translation is different in transformed and normal cells. Thus, it is possible that alterations at the translational level can in part account for the discrepancy between *HER2/neu* gene amplification detected by fluorescence *in situ* hybridization and protein levels detected by immunohistochemical assays.

Translation Targets of Selected Cancer Therapy

Components of the translation machinery and signal pathways involved in the activation of translation initiation represent good targets for cancer therapy.

Targeting the mTOR Signaling Pathway: Rapamycin and Temstatin

Rapamycin inhibits the proliferation of lymphocytes. It was initially developed as an immunosuppressive drug for organ

transplantation. Rapamycin with FKBP 12 (FK506-binding protein, *M*, 12,000) binds to mTOR to inhibit its function.

Rapamycin causes a small but significant reduction in the initiation rate of protein synthesis (83). It blocks cell growth in part by blocking S6 phosphorylation and selectively suppressing the translation of 5' TOP mRNAs, such as ribosomal proteins, and elongation factors (83–85). Rapamycin also blocks 4E-BP1 phosphorylation and inhibits cap-dependent but not cap-independent translation (17, 86).

The rapamycin-sensitive signal transduction pathway, activated during malignant transformation and cancer progression, is now being studied as a target for cancer therapy (87). Prostate, breast, small cell lung, glioblastoma, melanoma, and T-cell leukemia are among the cancer lines most sensitive to the rapamycin analogue CCI-779 (Wyeth-Ayerst Research; Ref. 87). In rhabdomyosarcoma cell lines, rapamycin is either cytostatic or cytotoxic, depending on the p53 status of the cell; p53 wild-type cells treated with rapamycin arrest in the G₁ phase and maintain their viability, whereas p53 mutant cells accumulate in G₁ and undergo apoptosis (88, 89). In a recently reported study using human primitive neuroectodermal tumor and medulloblastoma models, rapamycin exhibited more cytotoxicity in combination with cisplatin and camptothecin than as a single agent. *In vivo*, CCI-779 delayed growth of xenografts by 160% after 1 week of therapy and 240% after 2 weeks. A single high-dose administration caused a 37% decrease in tumor volume. Growth inhibition *in vivo* was 1.3 times greater, with cisplatin in combination with CCI-779 than with cisplatin alone (90). Thus, preclinical studies suggest that rapamycin analogues are useful as single agents and in combination with chemotherapy.

Rapamycin analogues CCI-779 and RAD001 (Novartis, Basel, Switzerland) are now in clinical trials. Because of the known effect of rapamycin on lymphocyte proliferation, a potential problem with rapamycin analogues is immunosuppression. However, although prolonged immunosuppression can result from rapamycin and CCI-779 administered on continuous-dose schedules, the immunosuppressive effects of rapamycin analogues resolve in ~24 h after therapy (91). The principal toxicities of CCI-779 have included dermatological toxicity, myelosuppression, infection, mucositis, diarrhea, reversible elevations in liver function tests, hyperglycemia, hypokalemia, hypocalcemia, and depression (87, 92–94). Phase II trials of CCI-779 have been conducted in advanced renal cell carcinoma and in stage III/IV breast carcinoma patients who failed with prior chemotherapy. In the results reported in abstract form, although there were no complete responses, partial responses were documented in both renal cell carcinoma and in breast carcinoma (94, 95). Thus, CCI-779 has documented preliminary clinical activity in a previously treated, unselected patient population.

Active investigation is under way into patient selection for mTOR inhibitors. Several studies have found an enhanced efficacy of CCI-779 in *PTEN*-null tumors (30, 96). Another study found that six of eight breast cancer cell lines were responsive to CCI-779, although only two of these lines lacked *PTEN* (97). There was, however, a positive correlation between Akt activation and CCI-779 sensitivity (97). This correlation suggests that activation of the PI3K-Akt pathway,

regardless of whether it is attributable to a PTEN mutation or to overexpression of receptor tyrosine kinases, makes cancer cell amenable to mTOR-directed therapy. In contrast, lower levels of the target of mTOR, 4E-BP1, are associated with rapamycin resistance; thus, a lower 4E-BP1/eIF4E ratio may predict rapamycin resistance (98).

Another mode of activity for rapamycin and its analogues appears to be through inhibition of angiogenesis. This activity may be both through direct inhibition of endothelial cell proliferation as a result of mTOR inhibition in these cells or by inhibition of translation of such proangiogenic factors as vascular endothelial growth factor in tumor cells (99, 100).

The angiogenesis inhibitor turostatin, another anticancer drug currently under study, was also found recently to inhibit translation in endothelial cells (101). Through a requisite interaction with Integrin, turostatin inhibits activation of the PI3K/Akt pathway and mTOR in endothelial cells and prevents dissociation of eIF4E from 4E-BP1, thereby inhibiting cap-dependent translation. These findings suggest that endothelial cells are especially sensitive to therapies targeting the mTOR-signaling pathway.

Targeting eIF2 α EPA, Clotrimazole, mda-7, and Flavonoids

EPA is an n-3 polyunsaturated fatty acid found in the fish-based diets of populations having a low incidence of cancer (102). EPA inhibits the proliferation of cancer cells (103), as well as in animal models (104, 105). It blocks cell division by inhibiting translation initiation (105). EPA releases Ca^{2+} from intracellular stores while inhibiting their refilling, thereby activating PKR. PKR, in turn phosphorylates and inhibits eIF2 α , resulting in the inhibition of protein synthesis at the level of translation initiation. Similarly, clotrimazole, a potent antiproliferative agent *in vitro* and *in vivo*, inhibits cell growth through depletion of Ca^{2+} stores, activation of PKR, and phosphorylation of eIF2 α (106). Consequently, clotrimazole preferentially decreases the expression of cyclins A, E, and D1, resulting in blockage of the cell cycle in G₁.

mda-7 is a novel tumor suppressor gene being developed as a gene therapy agent. Adenoviral transfer of mda-7 (Ad-mda7) induces apoptosis in many cancer cells including breast, colorectal, and lung cancer (107–109). Ad-mda7 also induces and activates PKR, which leads to phosphorylation of eIF2 α and induction of apoptosis (110).

Flavonoids such as genistein and quercetin suppress tumor cell growth. All three mammalian eIF2 α kinases, PKR, heme-regulated inhibitor, and PERK/PEK, are activated by flavonoids, with phosphorylation of eIF2 α and inhibition of protein synthesis (111).

Targeting eIF4A and eIF4E: Antisense RNA and Peptides

Antisense expression of eIF4A decreases the proliferation rate of melanoma cells (112). Sequestration of eIF4E by overexpression of 4E-BP1 is proapoptotic and decreases tumorigenicity (113, 114). Reduction of eIF4E with antisense RNA decreases soft agar growth, increases tumor latency, and increases the rates of tumor doubling times (7). Antisense eIF4E RNA treat-

ment also reduces the expression of angiogenic factors (115) and has been proposed as a potential adjuvant therapy for head and neck cancers, particularly when elevated eIF4E is found in surgical margins. Small molecule inhibitors that bind the eIF4G/4E-BP1-binding domain of eIF4E are proapoptotic (116) and are also being actively pursued.

Exploiting Selective Translation for Gene Therapy

A different therapeutic approach that takes advantage of the enhanced cap-dependent translation in cancer cells is the use of gene therapy vectors encoding suicide genes with highly structured 5' UTR. These mRNA would thus be at a competitive disadvantage in normal cells and not translate well, whereas in cancer cells, they would translate more efficiently. For example, the introduction of the 5' UTR of fibroblast growth factor-2 5' to the coding sequence of *herpes simplex virus type-1 thymidine kinase* gene, allows for selective translation of *herpes simplex virus type-1 thymidine kinase* gene in breast cancer cell lines compared with normal mammary cell lines and results in selective sensitivity to ganciclovir (117).

Toward the Future

Translation is a crucial process in every cell. However, several alterations in translational control occur in cancer. Cancer cells appear to need an aberrantly activated translational state for survival, thus allowing the targeting of translation initiation with surprisingly low toxicity. Components of the translational machinery, such as eIF4E, and signal transduction pathways involved in translation initiation, such as mTOR, represent promising targets for cancer therapy. Inhibitors of the mTOR have already shown some preliminary activity in clinical trials. It is possible that with the development of better predictive markers and better patient selection, response rates to single-agent therapy can be improved. Similar to other cytostatic agents, however, mTOR inhibitors are most likely to achieve clinical utility in combination therapy. In the interim, our increasing understanding of translation initiation and signal transduction pathways promise to lead to the identification of new therapeutic targets in the near future.

Acknowledgments

We thank Gayle Nesom from The University of Texas M. D. Anderson Cancer Center Department of Scientific Publications for editorial assistance and Dr. Elmer Bernstein for assistance with manuscript preparation.

References

1. Pestova, T. V., Kolupaeva, V. G., Lomakin, I. B., Pălpenko, E. V., Shatsky, I. N., Agol, V. I., and Hellen, C. U. Molecular mechanisms of translation initiation in eukaryotes. *Proc. Natl. Acad. Sci. USA*, 98: 7029–7036, 2001.
2. Rosenwald, I. B., Kaspar, R., Rousseau, D., Gehrke, L., Leboucq, P., Chen, J. J., Schmidt, E. V., Sonenberg, N., and London, I. M. Eukaryotic translation initiation factor 4E regulates expression of cyclin D1 at transcriptional and post-transcriptional levels. *J. Biol. Chem.*, 270: 21176–21180, 1995.
3. Rosenwald, I. B., Hutzler, M. J., Wang, S., Savas, L., and Fraire, A. E. Expression of eukaryotic translation initiation factors 4E and 2 α is increased frequently in bronchioloalveolar but not in squamous cell carcinomas of the lung. *Cancer (Phila.)*, 92: 2164–2171, 2001.

4. Darveau, A., Pelletier, J., and Sonenberg, N. Differential efficiencies of *in vitro* translation of mouse c-myc transcripts differing in the 5' untranslated region. *Proc. Natl. Acad. Sci. USA*, 82: 2316-2319, 1985.
5. Kozak, M. Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. *Proc. Natl. Acad. Sci. USA*, 83: 2850-2854, 1986.
6. Koromilas, A. E., Lazaris-Karatzas, A., and Sonenberg, N. mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E. *EMBO J.*, 11: 4153-4158, 1992.
7. Rinker-Schaeffer, C. W., Graff, J. R., De Benedetti, A., Zimmer, S. G., and Rhoads, R. E. Decreasing the level of translation initiation factor 4E with antisense RNA causes reversal of ras-mediated transformation and tumorigenesis of cloned rat embryo fibroblasts. *Int. J. Cancer*, 55: 841-847, 1993.
8. Kevi, C. G., De Benedetti, A., Payne, D. K., Coe, L. L., Laroux, F. S., and Alexander, J. S. Translational regulation of vascular permeability factor by eukaryotic initiation factor 4E: Implications for tumor angiogenesis. *Int. J. Cancer*, 65: 785-790, 1996.
9. Nathan, C. A., Franklin, S., Abreo, F. W., Nassar, R., De Benedetti, A., and Glass, J. Analysis of surgical margins with the molecular marker eIF4E: a prognostic factor in patients with head and neck cancer. *J. Clin. Oncol.*, 17: 2809-2814, 1999.
10. Fukunaga, R., and Hunter, T. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.*, 16: 1921-1933, 1997.
11. Waskiewicz, A. J., Flynn, A., Proud, C. G., and Cooper, J. A. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J.*, 16: 1909-1920, 1997.
12. Wang, X., Flynn, A., Waskiewicz, A. J., Webb, B. L., Vries, R. G., Balnes, L. A., Cooper, J. A., and Proud, C. G. The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. *J. Biol. Chem.*, 273: 8373-8377, 1998.
13. Pyronnet, S., Imataka, H., Gingras, A. C., Fukunaga, R., Hunter, T., and Sonenberg, N. Human eukaryotic translation initiation factor 4G (eIF4G) recruits Mnk1 to phosphorylate eIF4E. *EMBO J.*, 18: 270-279, 1999.
14. Klein, M., Scheper, G. C., Voorma, H. O., and Thomas, A. A. Regulation of translation initiation factors by signal transduction. *Eur. J. Biochem.*, 253: 531-544, 1998.
15. Raught, B., and Gingras, A. C. eIF4E activity is regulated at multiple levels. *Int. J. Biochem. Cell Biol.*, 31: 43-57, 1999.
16. Takeuchi, K., Shibamoto, S., Nagamine, K., Shigemori, I., Omura, S., Kitamura, N., and Ito, F. Signaling pathways leading to transcription and translation cooperatively regulate the transient increase in expression of c-Fos protein. *J. Biol. Chem.*, 276: 26077-26083, 2001.
17. Kawasome, H., Papst, P., Webb, S., Keller, G. M., Johnson, G. L., Gelfand, E. W., and Terada, N. Targeted disruption of p70(S6K) defines its role in protein synthesis and rapamycin sensitivity. *Proc. Natl. Acad. Sci. USA*, 95: 5033-5038, 1998.
18. Christie, G. R., Hajdich, E., Hundal, H. S., Proud, C. G., and Taylor, P. M. Intracellular sensing of amino acids in *Xenopus laevis* oocytes stimulates p70 S6 kinase in a target of rapamycin-dependent manner. *J. Biol. Chem.*, 277: 8952-8957, 2002.
19. Hara, K., Yonezawa, K., Weng, Q. P., Kozlowski, M. T., Belham, C., and Avruch, J. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J. Biol. Chem.*, 273: 14484-14494, 1998.
20. Graves, L. M., Bornfeldt, K. E., Argast, G. M., Krebs, E. G., Kong, X., Lin, T. A., and Lawrence, J. C., Jr. cAMP- and rapamycin-sensitive regulation of the association of eukaryotic initiation factor 4E and the translational regulator PHAS-I in aortic smooth muscle cells. *Proc. Natl. Acad. Sci. USA*, 92: 7222-7226, 1995.
21. Merrick, W. C., and Hershey, J. W. B. The pathway and mechanism of eukaryotic protein synthesis. In: J. W. B. Hershey and M. B. Mathews (eds.), *Translational Control*, pp. 31-69. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1986.
22. Kimball, S. R. Eukaryotic initiation factor eIF2. *Int. J. Biochem. Cell Biol.*, 31: 25-29, 1999.
23. Jagus, R., Joshi, B., and Barber, G. N. PKR, apoptosis and cancer. *Int. J. Biochem. Cell Biol.*, 31: 123-138, 1999.
24. Thomas, G., and Hall, M. N. TOR signalling and control of cell growth. *Curr. Opin. Cell Biol.*, 9: 782-787, 1997.
25. Gingras, A. C., Raught, B., and Sonenberg, N. Regulation of translation initiation by FRAP/mTOR. *Genes Dev.*, 15: 807-828, 2001.
26. Gingras, A. C., Gygi, S. P., Raught, B., Polakiewicz, R. D., Abraham, R. T., Hoekstra, M. F., Aebersold, R., and Sonenberg, N. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev.*, 13: 1422-1437, 1999.
27. Kumar, V., Pandey, P., Sabatini, D., Kumar, M., Majumder, P. K., Bharti, A., Carmichael, G., Kufe, D., and Karbhanda, S. Functional interaction between RAFT1/FRAP/mTOR and protein kinase C δ in the regulation of cap-dependent initiation of translation. *EMBO J.*, 19: 1087-1097, 2000.
28. Yang, D. Q., and Kastan, M. B. Participation of ATM in insulin signaling through phosphorylation of eIF-4E-binding protein 1. *Nat. Cell Biol.*, 2: 893-898, 2000.
29. Liu, G., Zhang, Y., Bode, A. M., Ma, W. Y., and Dong, Z. Phosphorylation of 4E-BP1 is mediated by the p38/MSK1 pathway in response to UVB irradiation. *J. Biol. Chem.*, 277: 8810-8818, 2002.
30. Neshat, M. S., Mellingerhoff, I. K., Tran, C., Stiles, B., Thomas, G., Petersen, R., Frost, P., Gibbons, J. J., Wu, H., and Sawyers, C. L. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. *Proc. Natl. Acad. Sci. USA*, 98: 10314-10319, 2001.
31. Sekulic, A., Hudson, C. C., Homme, J. L., Yin, P., Ottemess, D. M., Karnitz, L. M., and Abraham, R. T. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. *Cancer Res.*, 60: 3504-3513, 2000.
32. Scott, P. H., and Lawrence, J. C., Jr. Attenuation of mammalian target of rapamycin activity by increased cAMP in 3T3-L1 adipocytes. *J. Biol. Chem.*, 273: 34496-34501, 1998.
33. Reynolds, I. T., Bodine, S. C., and Lawrence, J. C., Jr. Control of Ser2448 phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load. *J. Biol. Chem.*, 277: 17657-17662, 2002.
34. Peterson, R. T., Beal, P. A., Comb, M. J., and Schreiber, S. L. FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. *J. Biol. Chem.*, 275: 7416-7423, 2000.
35. Peterson, R. T., Desai, B. N., Hardwick, J. S., and Schreiber, S. L. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycin-associated protein. *Proc. Natl. Acad. Sci. USA*, 96: 4438-4442, 1999.
36. McGrew, L. L., Dworkin-Rastl, E., Dworkin, M. B., and Richter, J. D. Poly(A) elongation during *Xenopus* oocyte maturation is required for translational recruitment and is mediated by a short sequence element. *Genes Dev.*, 3: 803-815, 1989.
37. Sheets, M. D., Wu, M., and Wickens, M. Polyadenylation of c-mos mRNA as a control point in *Xenopus* meiotic maturation. *Nature (Lond.)*, 374: 511-516, 1995.
38. Varnum, S. M., and Wormington, W. M. Deadenylation of maternal mRNAs during *Xenopus* oocyte maturation does not require specific cis-sequences: a default mechanism for translational control. *Genes Dev.*, 4: 2278-2286, 1990.
39. Gallie, D. R. The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. *Genes Dev.*, 5: 2108-2116, 1991.
40. Sachs, A. B., and Varani, G. Eukaryotic translation initiation: there are (at least) two sides to every story. *Nat. Struct. Biol.*, 7: 356-361, 2000.
41. Wolffe, A. P., and Merlo, F. Coupling transcription to translation: a novel site for the regulation of eukaryotic gene expression. *Int. J. Biochem. Cell Biol.*, 28: 247-257, 1996.
42. Evdokimova, V. M., Wei, C. L., Sitikov, A. S., Simonenko, P. N., Lazarev, O. A., Vasilenko, K. S., Ustinov, V. A., Hershey, J. W., and Ovchinnikov, L. P. The major protein of messenger ribonucleoprotein particles in somatic cells is a member of the Y-box binding transcription factor family. *J. Biol. Chem.*, 270: 3186-3192, 1995.
43. Matsumoto, K., Merlo, F., and Wolffe, A. P. Translational repression dependent on the interaction of the *Xenopus* Y-box protein FRGY2 with mRNA. Role of the cold shock domain, tail domain, and selective RNA sequence recognition. *J. Biol. Chem.*, 271: 22706-22712, 1996.

44. Evdokimova, V., Ruzanov, P., Imataka, H., Raught, B., Svitkin, Y., Ovchinnikov, L. P., and Sonenberg, N. The major mRNA-associated protein YB-1 is a potent 5' cap-dependent mRNA stabilizer. *EMBO J.*, 20: 5491-5502, 2001.
45. Saito, H., Hayday, A. C., Wiman, K., Hayward, W. S., and Tonegawa, S. Activation of the *c-myc* gene by translocation: a model for translational control. *Proc. Natl. Acad. Sci. USA*, 80: 7476-7480, 1983.
46. Nanbru, C., Lafon, I., Audigier, S., Gensac, M. C., Vagner, S., Huez, G., and Prats, A. C. Alternative translation of the proto-oncogene *c-myc* by an internal ribosome entry site. *J. Biol. Chem.*, 272: 32061-32066, 1997.
47. Stoneley, M., Paulin, F. E., Le Quesne, J. P., Chappell, S. A., and Willis, A. E. *c-Myc* 5' untranslated region contains an internal ribosome entry segment. *Oncogene*, 16: 423-428, 1998.
48. Paulin, F. E., West, M. J., Sullivan, N. F., Whitney, R. L., Lyne, L., and Willis, A. E. Aberrant translational control of the *c-myc* gene in multiple myeloma. *Oncogene*, 13: 505-513, 1996.
49. Chappell, S. A., LeQuesne, J. P., Paulin, F. E., de Schootmeester, M. L., Stoneley, M., Soutar, R. L., Ralston, S. H., Helfrich, M. H., and Willis, A. E. A mutation in the *c-myc*-IRES leads to enhanced internal ribosome entry in multiple myeloma: a novel mechanism of oncogene de-regulation. *Oncogene*, 19: 4437-4440, 2000.
50. Signori, E., Bagri, C., Papa, S., Primerano, B., Rinaldi, M., Amaldi, F., and Fazio, V. M. A somatic mutation in the 5'UTR of *BRCA1* gene in sporadic breast cancer causes down-modulation of translation efficiency. *Oncogene*, 20: 4598-4600, 2001.
51. Liu, L., Diworth, D., Gao, L., Morzon, J., Summers, A., Lassam, N., and Hogg, D. Mutation of the *CDKN2A* 5' UTR creates an aberrant initiation codon and predisposes to melanoma. *Nat. Genet.*, 21: 128-132, 1999.
52. Savitsky, K., Platzer, M., Uziel, T., Gilad, S., Sarti, A., Rosenthal, A., Eroy-Stein, O., Shilo, Y., and Rotman, G. Ataxia-telangiectasia: structural diversity of untranslated sequences suggests complex post-transcriptional regulation of *ATM* gene expression. *Nucleic Acids Res.*, 25: 1678-1684, 1997.
53. Brown, C. Y., Mize, G. J., Pineda, M., George, D. L., and Morris, D. R. Role of two upstream open reading frames in the translational control of oncogene *mdm2*. *Oncogene*, 18: 5631-5637, 1999.
54. Sobczak, K., and Krzyzosiak, W. J. Structural determinants of *BRCA1* translational regulation. *J. Biol. Chem.*, 277: 17349-17358, 2002.
55. Arrick, B. A., Grendell, R. L., and Griffin, L. A. Enhanced translational efficiency of a novel transforming growth factor β mRNA in human breast cancer cells. *Mol. Cell. Biol.*, 14: 619-628, 1994.
56. Myers, S. M., Eng, C., Ponder, B. A., and Mulligan, L. M. Characterization of *RET* proto-oncogene 3' splicing variants and polyadenylation sites: a novel C-terminus for *RET*. *Oncogene*, 11: 2039-2045, 1995.
57. Byrne, J. A., Tomasello, C., Rouyer, N., Bellocq, J. P., Rio, M. C., and Basset, P. The tissue inhibitor of metalloproteinases-3 gene in breast carcinoma: identification of multiple polyadenylation sites and a stromal pattern of expression. *Mol. Med.*, 1: 418-427, 1995.
58. Moscow, J. A., He, R., Gudas, J. M., and Cowan, K. H. Utilization of multiple polyadenylation signals in the human *RHOA* protooncogene. *Gene (Amst.)*, 144: 229-236, 1994.
59. Senterre-Lesentants, S., Alag, A. S., and Sobel, M. E. Multiple mRNA species are generated by alternate polyadenylation from the human *calmodulin-1* gene. *J. Cell. Biochem.*, 58: 445-454, 1995.
60. Lazaris-Karatzas, A., Montine, K. S., and Sonenberg, N. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature (Lond.)*, 345: 544-547, 1990.
61. De Benedetti, A., and Rhoads, R. E. Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology. *Proc. Natl. Acad. Sci. USA*, 87: 8212-8216, 1990.
62. Polunovsky, V. A., Rosenwald, I. B., Tan, A. T., White, J., Chiang, L., Sonenberg, N., and Blitman, P. B. Translational control of programmed cell death: eukaryotic translation initiation factor 4E blocks apoptosis in growth-factor-restricted fibroblasts with physiologically expressed or de-regulated *Myc*. *Mol. Cell. Biol.*, 16: 6573-6581, 1996.
63. Miyagi, Y., Sugiyama, A., Asai, A., Okazaki, T., Kuchino, Y., and Kerr, S. J. Elevated levels of eukaryotic translation initiation factor eIF-4E mRNA in a broad spectrum of transformed cell lines. *Cancer Lett.*, 91: 247-252, 1995.
64. Kerekatte, V., Smiley, K., Hu, B., Smith, A., Gelder, F., and De Benedetti, A. The proto-oncogene/translation factor eIF4E: a survey of its expression in breast carcinomas. *Int. J. Cancer*, 64: 27-31, 1995.
65. Li, B. D., Liu, L., Dawson, M., and De Benedetti, A. Overexpression of eukaryotic initiation factor 4E (eIF4E) in breast carcinoma. *Cancer (Phila.)*, 79: 2385-2390, 1997.
66. Sorrelle, D. L., Black, D. R., Meschonat, C., Rhoads, R., De Benedetti, A., Gao, M., Williams, B. J., and Li, B. D. Detection of eIF4E gene amplification in breast cancer by competitive PCR. *Ann. Surg. Oncol.*, 5: 232-237, 1998.
67. Li, B. D., McDonald, J. C., Nassar, R., and De Benedetti, A. Clinical outcome in stage I to III breast carcinoma and eIF4E overexpression. *Ann. Surg.*, 227: 756-761; discussion, 761-763, 1998.
68. Wang, S., Rosenwald, I. B., Hutzler, M. J., Pihan, G. A., Savas, L., Chen, J. J., and Woda, B. A. Expression of the eukaryotic translation initiation factors 4E and 2a in non-Hodgkin's lymphomas. *Am. J. Pathol.*, 155: 247-255, 1999.
69. Fukuchi-Shimogori, T., Ishii, I., Kashiwagi, K., Mashiba, H., Ekimoto, H., and Igarashi, K. Malignant transformation by overproduction of translation initiation factor eIF4G. *Cancer Res.*, 57: 5041-5044, 1997.
70. Eberle, J., Krasagakis, K., and Orfanos, C. E. Translation initiation factor eIF-4A1 mRNA is consistently overexpressed in human melanoma cells *in vitro*. *Int. J. Cancer*, 71: 396-401, 1997.
71. Shuda, M., Kondoh, N., Tanaka, K., Ryo, A., Wakatsuki, T., Hada, A., Goseki, N., Igarashi, T., Hatsuse, K., Aihara, T., Horuchi, S., Shichita, M., Yamamoto, N., and Yamamoto, M. Enhanced expression of translation factor mRNAs in hepatocellular carcinoma. *Anticancer Res.*, 20: 2489-2494, 2000.
72. Nupponen, N. N., Porkka, K., Kakkola, L., Tanner, M., Persson, K., Borg, A., Isola, J., and Visakorpi, T. Amplification and overexpression of p40 subunit of eukaryotic translation initiation factor 3 in breast and prostate cancer. *Am. J. Pathol.*, 154: 1777-1783, 1999.
73. Roth, M., Ko, Y., Aibers, P., and Wernert, N. Eukaryotic initiation factor 3 p110 mRNA is overexpressed in testicular seminomas. *Am. J. Pathol.*, 157: 1597-1604, 2000.
74. Barlund, M., Forozan, F., Kononen, J., Bubendorf, L., Chen, Y., Blitner, M. L., Torhorst, J., Haas, P., Bucher, C., Sauter, G., Kallioniemi, O. P., and Kallioniemi, A. Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. *J. Natl. Cancer Inst. (Bethesda)*, 92: 1252-1259, 2000.
75. Topalian, S. L., Kaneko, S., Gonzales, M. I., Bond, G. L., Ward, Y., and Manley, J. L. Identification and functional characterization of neo-poly(A) polymerase, an RNA processing enzyme overexpressed in human tumors. *Mol. Cell. Biol.*, 21: 5614-5623, 2001.
76. Scorilas, A., Tallier, M., Ardavanis, A., Courtis, N., Dimitriadis, E., Yotis, J., Tsalpalis, C. M., and Trangas, T. Polyadenylate polymerase enzymatic activity in mammary tumor cytosols: a new independent prognostic marker in primary breast cancer. *Cancer Res.*, 60: 5427-5433, 2000.
77. Janz, M., Harbeck, N., Dettmar, P., Berger, U., Schmidt, A., Jurchott, K., Schmitt, M., and Royer, H. D. Y-box factor YB-1 predicts drug resistance and patient outcome in breast cancer independent of clinically relevant tumor biologic factors HER2, uPA and PAI-1. *Int. J. Cancer*, 97: 278-282, 2002.
78. Shibahara, K., Sugio, K., Osaki, T., Uchiumi, T., Maehara, Y., Kohno, K., Yasumoto, K., Sugimachi, K., and Kuwano, M. Nuclear expression of the Y-box binding protein, YB-1, as a novel marker of disease progression in non-small cell lung cancer. *Clin. Cancer Res.*, 7: 3151-3155, 2001.
79. Kamura, T., Yahata, H., Amada, S., Ogawa, S., Sonoda, T., Kobayashi, H., Mitsumoto, M., Kohno, K., Kuwano, M., and Nakano, H. Is nuclear expression of Y box-binding protein-1 a new prognostic factor in ovarian serous adenocarcinoma? *Cancer (Phila.)*, 85: 2450-2454, 1999.
80. Bargou, R. C., Jurchott, K., Wagener, C., Bergmann, S., Metzner, S., Bommer, K., Mapara, M. Y., Winzer, K. J., Dietel, M., Dorkan, B., and Royer, H. D. Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic *MDR1* gene expression. *Nat. Med.*, 3: 447-450, 1997.
81. Aoki, M., Blazek, E., and Vogt, P. K. A role of the kinase mTOR in cellular transformation induced by the oncoproteins P3K and Akt. *Proc. Natl. Acad. Sci. USA*, 98: 136-141, 2001.
82. Child, S. J., Miller, M. K., and Geballe, A. P. Cell type-dependent and -independent control of HER-2/neu translation. *Int. J. Biochem. Cell Biol.*, 31: 201-213, 1999.

83. Jefferies, H. B., Reinhard, C., Kozma, S. C., and Thomas, G. Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. *Proc. Natl. Acad. Sci. USA*, 91: 4441-4445, 1994.
84. Terada, N., Patel, H. R., Takase, K., Kohno, K., Naim, A. C., and Gelfand, E. W. Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. *Proc. Natl. Acad. Sci. USA*, 91: 11477-11481, 1994.
85. Jefferies, H. B., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B., and Thomas, G. Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. *EMBO J.*, 16: 3693-3704, 1997.
86. Beretta, L., Gingras, A. C., Svitkin, Y. V., Hall, M. N., and Sonenberg, N. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *EMBO J.*, 15: 658-664, 1996.
87. Hidalgo, M., and Rowinsky, E. K. The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. *Oncogene*, 19: 6880-6886, 2000.
88. Hosoi, H., Dilling, M. B., Shikata, T., Liu, L. N., Shu, L., Ashmun, R. A., Germain, G. S., Abraham, R. T., and Houghton, P. J. Rapamycin causes poorly reversible inhibition of mTOR and induces p53-independent apoptosis in human rhabdomyosarcoma cells. *Cancer Res.*, 59: 888-894, 1999.
89. Huang, S., and Houghton, P. J. Resistance to rapamycin: a novel anticancer drug. *Cancer Metastasis Rev.*, 20: 69-78, 2001.
90. Georger, B., Kerr, K., Tang, C. B., Fung, K. M., Powell, B., Sutton, L. N., Phillips, P. C., and Janss, A. J. Antitumor activity of the rapamycin analog CCI-779 in human primitive neuroectodermal tumor/medulloblastoma models as single agent and in combination chemotherapy. *Cancer Res.*, 61: 1527-1532, 2001.
91. Gibbons, J. J., Discafani, C., Peterson, R., Hernandez, R., Skotnick, J., and Frost, P. The effect of CCI-779, a novel macrolide anti-tumor agent, on the growth of human tumor cells *in vitro* and in nude mouse xenografts *in vivo*. *Proc. Am. Assoc. Cancer Res.*, 40: 301, 1999.
92. Hidalgo, M., Rowinsky, E., Erlichman, C., Marshall, B., Marks, R., Edwards, T., and Buckner, J. J. A Phase I and pharmacological study of CCI-779 cycle inhibitor. *Ann. Oncol.*, 11 (Suppl. 4): 133, 2001.
93. Alexandre, J., Raymond, E., Depenbrock, H., Mekhaldi, S., Angevin, E., Paillet, C., Hanauska, A., Frisch, J., Feussner, A., and Armand, J. P. CCI-779, a new rapamycin analog, has antitumor activity at doses inducing only mild cutaneous effects and mucositis: early results of an ongoing Phase I study. *Proceedings of the 1999 AACR-NCI-EORTC International Conference, Clin. Cancer Res.*, 5 (Suppl.): 3730s, 1999.
94. Chan, S., Johnston, S., Scheulen, M. E., Mross, K., Morant, A., Lehr, A., Feussner, A., Berger, M., and Kirsch, T. First report: a Phase 2 study of the safety and activity of CCI-779 for patients with locally advanced or metastatic breast cancer failing prior chemotherapy. *Proc. Am. Soc. Clin. Oncol.*, 21: 44a, 2002.
95. Atkins, M. B., Hidalgo, M., Stadler, W., Logan, T., Dutcher, J. P., Hudes, G., Park, Y., Marshall, B., Boni, J., and Dukart, G. A randomized double-blind Phase 2 study of intravenous CCI-779 administered weekly to patients with advanced renal cell carcinoma. *Proc. Am. Soc. Clin. Oncol.*, 21: 10a, 2002.
96. Smith, S. G., Trinh, C. M., Inge, L. J., Thomas, G., Cloughsey, T. F., Sawyers, C. L., and Mischel, P. S. PTEN expression status predicts glioblastoma cell sensitivity to CCI-779. *Proc. Am. Assoc. Cancer Res.*, 43: 335, 2002.
97. Yu, K., Toral-Barza, L., Discafani, C., Zhang, W. G., Skotnick, J., Frost, P., and Gibbons, J. J. mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer. *Endocr. Relat. Cancer*, 8: 249-258, 2001.
98. Dilling, M. B., Germain, G. S., Dudkin, L., Jayaraman, A. L., Zhang, X., Harwood, F. C., and Houghton, P. J. 4E-binding proteins, the suppressors of eukaryotic initiation factor 4E, are downregulated in cells with acquired or intrinsic resistance to rapamycin. *J. Biol. Chem.*, 277: 13907-13917, 2002.
99. Guba, M., von Breitenbuch, P., Steinbauer, M., Koehl, G., Flagel, S., Homung, M., Bruns, C. J., Zuelke, C., Farkas, S., Anthuber, M., Jauch, K. W., and Gelssler, E. K. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat. Med.*, 8: 128-135, 2002.
100. Lane, H. A., Schell, C., Thauer, A., O'Reilly, T., and Wood, J. Anti-angiogenic activity of RAD001, an orally active anticancer agent. *Proc. Am. Assoc. Cancer Res.*, 43: 184, 2002.
101. Maeshima, Y., Sudhakar, A., Lively, J. C., Ueki, K., Kharbada, S., Kahn, C. R., Sonenberg, N., Hynes, R. O., and Kaluri, R. Tumorstatin, an endothelial cell-specific inhibitor of protein synthesis. *Science (Wash. DC)*, 295: 140-143, 2002.
102. Caygill, C. P., Charlett, A., and Hill, M. J. Fat, fish, fish oil and cancer. *Br. J. Cancer*, 74: 159-164, 1996.
103. Falconer, J. S., Ross, J. A., Fearon, K. C., Hawkins, R. A., O'Riordan, M. G., and Carter, D. C. Effect of eicosapentaenoic acid and other fatty acids on the growth *in vitro* of human pancreatic cancer cell lines. *Br. J. Cancer*, 69: 826-832, 1994.
104. Noguchi, M., Minami, M., Yagasaki, R., Kinoshita, K., Earashi, M., Kitagawa, H., Taniya, T., and Miyazaki, I. Chemoprevention of DMBA-induced mammary carcinogenesis in rats by low-dose EPA and DHA. *Br. J. Cancer*, 75: 348-353, 1997.
105. Palakurthi, S. S., Fluckiger, R., Aktas, H., Changolkar, A. K., Shahsafaee, A., Hamelt, S., Kilic, E., and Halperin, J. A. Inhibition of translation initiation mediates the anticancer effect of the n-3 polyunsaturated fatty acid eicosapentaenoic acid. *Cancer Res.*, 60: 2919-2925, 2000.
106. Aktas, H., Fluckiger, R., Acosta, J. A., Savage, J. M., Palakurthi, S. S., and Halperin, J. A. Depletion of intracellular Ca^{2+} stores, phosphorylation of eIF2 α , and sustained inhibition of translation initiation mediate the anticancer effects of clotrimazole. *Proc. Natl. Acad. Sci. USA*, 95: 8280-8285, 1998.
107. Mhashilkar, A. M., Schrock, R. D., Hindi, M., Liao, J., Sieger, K., Kourouma, F., Zou-Yang, X. H., Onishi, E., Takh, O., Vedvick, T. S., Fanger, G., Stewart, L., Watson, G. J., Snary, D., Fisher, P. B., Saeki, T., Roth, J. A., Ramesh, R., and Chada, S. Melanoma differentiation associated gene-7 (*mda-7*): a novel anti-tumor gene for cancer gene therapy. *Mol. Med.*, 7: 271-282, 2001.
108. Su, Z. Z., Madireddi, M. T., Lin, J. J., Young, C. S., Kitada, S., Reed, J. C., Goldstein, N. I., and Fisher, P. B. The cancer growth suppressor gene *mda-7* selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice. *Proc. Natl. Acad. Sci. USA*, 95: 14400-14405, 1998.
109. Saeki, T., Mhashilkar, A., Chada, S., Branch, C., Roth, J. A., and Ramesh, R. Tumor-suppressive effects by adenovirus-mediated *mda-7* gene transfer in non-small cell lung cancer cell *in vitro*. *Gene Ther.*, 7: 2051-2057, 2000.
110. Pataer, A., Vorburger, S. A., Barber, G. N., Chada, S., Mhashilkar, A. M., Zou-Yang, H., Stewart, A. L., Balachandran, S., Roth, J. A., Hunt, K. K., and Swisher, S. G. Adenoviral transfer of the melanoma differentiation-associated gene 7 (*mda7*) induces apoptosis of lung cancer cells via up-regulation of the double-stranded RNA-dependent protein kinase (PKR). *Cancer Res.*, 62: 2239-2243, 2002.
111. Ito, T., Wamken, S. P., and May, W. S. Protein synthesis inhibition by flavonoids: roles of eukaryotic initiation factor 2 α kinases. *Biochem. Biophys. Res. Commun.*, 265: 589-594, 1999.
112. Eberle, J., Fecker, L. F., Bittner, J. U., Orfanos, C. E., and Geilen, C. C. Decreased proliferation of human melanoma cell lines caused by antisense RNA against translation factor eIF-4A1. *Br. J. Cancer*, 86: 1957-1962, 2002.
113. Polunovsky, V. A., Gingras, A. C., Sonenberg, N., Peterson, M., Tan, A., Rubins, J. B., Manivel, J. C., and Bitterman, P. B. Translational control of the antiapoptotic function of Ras. *J. Biol. Chem.*, 275: 24778-24780, 2000.
114. D'Cunha, J., Kratzke, M. G., Alter, M. D., Polunovsky, V. A., Bitterman, P. B., and Kratzke, R. A. Over-expression of the translational repressor 4E-BP1 inhibits NSCLC tumorigenicity *in vivo*. *Proc. Am. Assoc. Cancer Res.*, 43: 816-817, 2002.
115. DeFatta, R. J., Nathan, C. A., and De Benedetti, A. Antisense RNA to eIF4E suppresses oncogenic properties of a head and neck squamous cell carcinoma cell line. *Laryngoscope*, 110: 828-833, 2000.
116. Herbert, T. P., Fahraeus, R., Prescott, A., Lane, D. P., and Proud, C. G. Rapid induction of apoptosis mediated by peptides that bind initiation factor eIF4E. *Curr. Biol.*, 10: 793-798, 2000.
117. DeFatta, R. J., Li, Y., and De Benedetti, A. Selective killing of cancer cells based on translational control of a suicide gene. *Cancer Gene Ther.*, 9: 573-578, 2002.

Correlation between Protein and mRNA Abundance in Yeast

STEVEN P. GYGI, YVAN ROCHON, B. ROBERT FRANZA, AND RUEDI AEBERSOLD*

Department of Molecular Biotechnology, University of Washington, Seattle, Washington 98195-7730

Received 5 October 1998/Returned for modification 11 November 1998/Accepted 2 December 1998

We have determined the relationship between mRNA and protein expression levels for selected genes expressed in the yeast *Saccharomyces cerevisiae* growing at mid-log phase. The proteins contained in total yeast cell lysate were separated by high-resolution two-dimensional (2D) gel electrophoresis. Over 150 protein spots were excised and identified by capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS). Protein spots were quantified by metabolic labeling and scintillation counting. Corresponding mRNA levels were calculated from serial analysis of gene expression (SAGE) frequency tables (V. E. Velculescu, L. Zhang, W. Zhou, J. Vogelstein, M. A. Basrai, D. E. Bassett, Jr., P. Hieter, B. Vogelstein, and K. W. Kinzler, Cell 88:243-251, 1997). We found that the correlation between mRNA and protein levels was insufficient to predict protein expression levels from quantitative mRNA data. Indeed, for some genes, while the mRNA levels were of the same value the protein levels varied by more than 20-fold. Conversely, invariant steady-state levels of certain proteins were observed with respective mRNA transcript levels that varied by as much as 30-fold. Another interesting observation is that codon bias is not a predictor of either protein or mRNA levels. Our results clearly delineate the technical boundaries of current approaches for quantitative analysis of protein expression and reveal that simple deduction from mRNA transcript analysis is insufficient.

The description of the state of a biological system by the quantitative measurement of the system constituents is an essential but largely unexplored area of biology. With recent technical advances including the development of differential display-PCR (21), of cDNA microarray and DNA chip technology (20, 27), and of serial analysis of gene expression (SAGE) (34, 35), it is now feasible to establish global and quantitative mRNA expression profiles of cells and tissues in species for which the sequence of all the genes is known. However, there is emerging evidence which suggests that mRNA expression patterns are necessary but are by themselves insufficient for the quantitative description of biological systems. This evidence includes discoveries of posttranscriptional mechanisms controlling the protein translation rate (15), the half-lives of specific proteins or mRNAs (33), and the intracellular location and molecular association of the protein products of expressed genes (32).

Proteome analysis, defined as the analysis of the protein complement expressed by a genome (26), has been suggested as an approach to the quantitative description of the state of a biological system by the quantitative analysis of protein expression profiles (36). Proteome analysis is conceptually attractive because of its potential to determine properties of biological systems that are not apparent by DNA or mRNA sequence analysis alone. Such properties include the quantity of protein expression, the subcellular location, the state of modification, and the association with ligands, as well as the rate of change with time of such properties. In contrast to the genomes of a number of microorganisms (for a review, see reference 11) and the transcriptome of *Saccharomyces cerevisiae* (35), which have been entirely determined, no proteome map has been completed to date.

The most common implementation of proteome analysis is the combination of two-dimensional gel electrophoresis (2DE)

(isoelectric focusing-sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis) for the separation and quantitation of proteins with analytical methods for their identification. 2DE permits the separation, visualization, and quantitation of thousands of proteins reproducibly on a single gel (18, 24). By itself, 2DE is strictly a descriptive technique. The combination of 2DE with protein analytical techniques has added the possibility of establishing the identities of separated proteins (1, 2) and thus, in combination with quantitative mRNA analysis, of correlating quantitative protein and mRNA expression measurements of selected genes.

The recent introduction of mass spectrometric protein analysis techniques has dramatically enhanced the throughput and sensitivity of protein identification to a level which now permits the large-scale analysis of proteins separated by 2DE. The techniques have reached a level of sensitivity that permits the identification of essentially any protein that is detectable in the gels by conventional protein staining (9, 29). Current protein analytical technology is based on the mass spectrometric generation of peptide fragment patterns that are idiotypic for the sequence of a protein. Protein identity is established by correlating such fragment patterns with sequence databases (10, 22, 37). Sophisticated computer software (8) has automated the entire process such that proteins are routinely identified with no human interpretation of peptide fragment patterns.

In this study, we have analyzed the mRNA and protein levels of a group of genes expressed in exponentially growing cells of the yeast *S. cerevisiae*. Protein expression levels were quantified by metabolic labeling of the yeast proteins to a steady state, followed by 2DE and liquid scintillation counting of the selected, separated protein species. Separated proteins were identified by in-gel tryptic digestion of spots with subsequent analysis by microspray liquid chromatography-tandem mass spectrometry (LC-MS/MS) and sequence database searching. The corresponding mRNA transcript levels were calculated from SAGE frequency tables (35).

This study, for the first time, explores a quantitative comparison of mRNA transcript and protein expression levels for a relatively large number of genes expressed in the same metabolic state. The resultant correlation is insufficient for predic-

* Corresponding author. Mailing address: Department of Molecular Biotechnology, Box 357730, University of Washington, Seattle, WA 98195-7730. Phone: (206) 221-4196. Fax: (206) 685-7301. E-mail: ruedi@u.washington.edu.

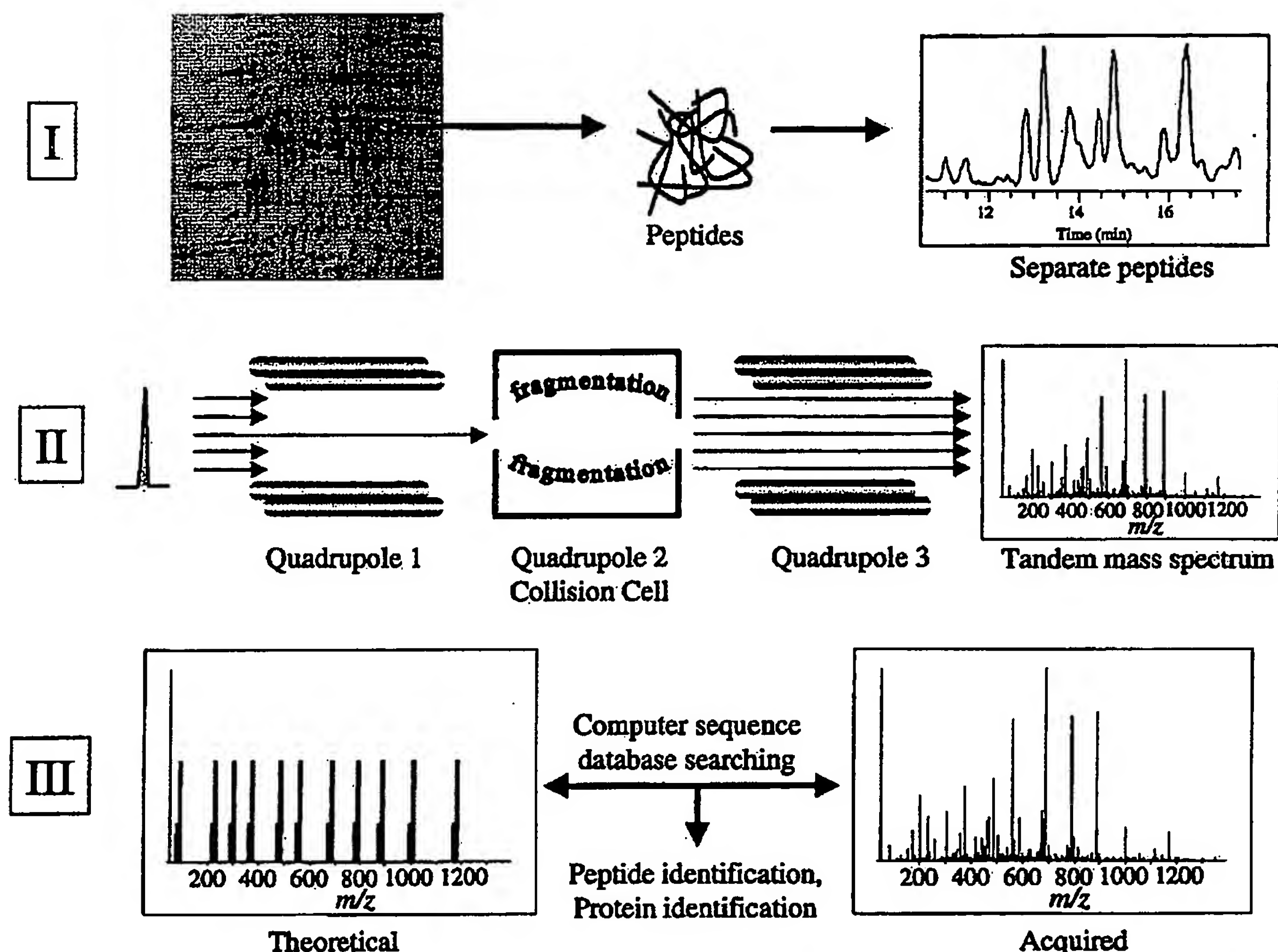


FIG. 1. Schematic illustration of proteome analysis by 2DE and mass spectrometry. In part I, proteins are separated by 2DE, stained spots are excised and subjected to in-gel digestion with trypsin, and the resulting peptides are separated by on-line capillary high-performance liquid chromatography. In part II, a peptide is shown eluting from the column in part I. The peptide is ionized by electrospray ionization and enters the mass spectrometer. The mass of the ionized peptide is detected, and the first quadrupole mass filter allows only the specific mass-to-charge ratio of the selected peptide ion to pass into the collision cell. In the collision cell, the energized, ionized peptides collide with neutral argon gas molecules. Fragmentation of the peptide is essentially random but occurs mainly at the peptide bonds, resulting in smaller peptides of differing lengths (masses). These peptide fragments are detected as a tandem mass (MS/MS) spectrum in the third quadrupole mass filter where two ion series are recorded simultaneously, one each from sequencing inward from the N and C termini of the peptide, respectively. In part III, the MS/MS spectrum from the selected, ionized peptide is compared to predicted tandem mass spectra computer generated from a sequence database. Provided that the peptide sequence exists in the database, the peptide and, by association, the protein from which the peptide was derived can be identified. Unambiguous protein identification is attained in a single analysis because multiple peptides are identified as being derived from the same protein.

tion of protein levels from mRNA transcript levels. We have also compared the relative amounts of protein and mRNA with the respective codon bias values for the corresponding genes. This comparison indicates that codon bias by itself is insufficient to accurately predict either the mRNA or the protein expression levels of a gene. In addition, the results demonstrate that only highly expressed proteins are detectable by 2DE separation of total cell lysates and that therefore the construction of complete proteome maps with current technology will be very challenging, irrespective of the type of organism.

MATERIALS AND METHODS

Yeast strain and growth conditions. The source of protein and message transcripts for all experiments was YPH499 (*MATa ura3-52 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 trp1-Δ63*) (30). Logarithmically growing cells were obtained by growing yeast cells to early log phase (3×10^6 cells/ml) in YPD rich medium (YPD supplemented with 6 mM uracil, 4.8 mM adenine, and 24 mM tryptophan) at 30°C (35). Metabolic labeling of protein was accomplished in YPD medium

exactly as described elsewhere (4) with the exception that 1 ml of cells was labeled with 3 mCi to offset methionine present in YPD medium. Protein was harvested as described by Garrels and coworkers (12). Harvested protein was lyophilized, resuspended in isoelectric focusing gel rehydration solution, and stored at -80°C.

2DE. Soluble proteins were run in the first dimension by using a commercial flatbed electrophoresis system (Multiphor II; Pharmacia Biotech). Immobilized polyacrylamide gel (IPG) dry strips with nonlinear pH 3.0 to 10.0 gradients (Amersham-Pharmacia Biotech) were used for the first-dimension separation. Forty micrograms of protein from whole-cell lysates was mixed with IPG strip rehydration buffer (8 M urea, 2% Nonidet P-40, 10 mM dithiothreitol), and 250 to 380 μ l of solution was added to individual lanes of an IPG strip rehydration tray (Amersham-Pharmacia Biotech). The strips were allowed to rehydrate at room temperature for 1 h. The samples were run at 300 V-10 mA-5 W for 2 h, then ramped to 3,500 V-10 mA-5 W over a period of 3 h, and then kept at 3,500 V-10 mA-5 W for 15 to 19 h. At the end of the first-dimension run (60 to 70 kV · h), the IPG strips were reequilibrated for 8 min in 2% (wt/vol) dithiothreitol in 2% (wt/vol) SDS-6 M urea-30% (wt/vol) glycerol-0.05 M Tris HCl (pH 6.8) and for 4 min in 2.5% iodoacetamide in 2% (wt/vol) SDS-6 M urea-30% (wt/vol) glycerol-0.05 M Tris HCl (pH 6.8). Following reequilibration, the strips were transferred and apposed to 10% polyacrylamide second-dimension gels. Polyacrylamide gels were poured in a casting stand with 10% acrylamide-2.67% piperazine diacrylamide-0.375 M Tris base-HCl (pH 8.8)-0.1% (wt/vol) SDS-0.05%

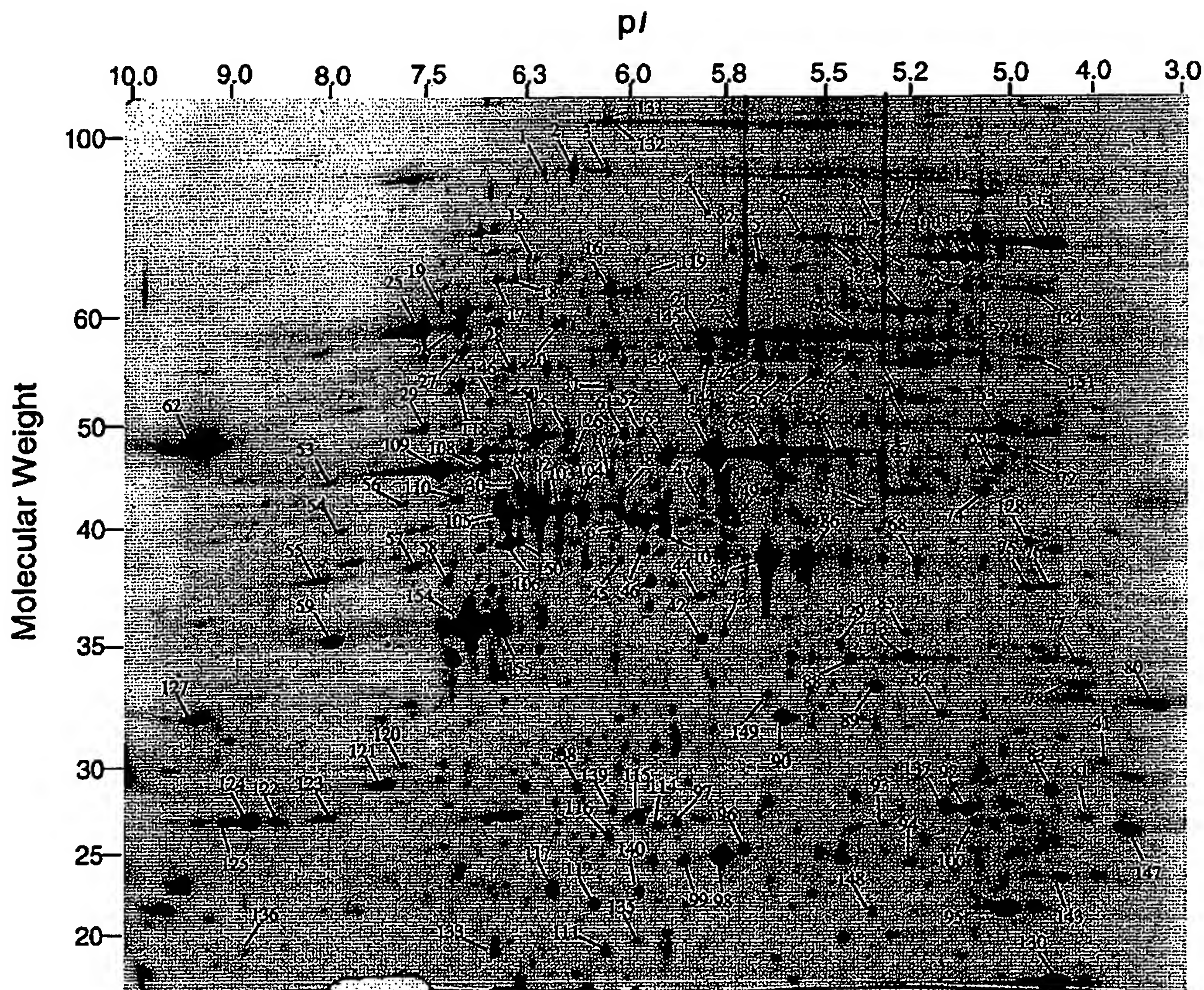


FIG. 2. 2D silver-stained gel of the proteins in yeast total cell lysate. Proteins were separated in the first dimension (horizontal) by isoelectric focusing and then in the second dimension (vertical) by molecular weight sieving. Protein spots (156) were chosen to include the entire range of molecular weights, isoelectric focusing points, and staining intensities. Spots were excised, and the corresponding protein was identified by mass spectrometry and database searching. The spots are labeled on the gel and correspond to the data presented in Table 1. Molecular weights are given in thousands.

(wt/vol) ammonium persulfate-0.05% TEMED (*N,N,N,N*-tetramethylethylenediamine) in Milli-Q water. The apparatus used to run second-dimension gels was a noncommercial apparatus from Oxford Glycosciences, Inc. Once the IPG strips were apposed to the second-dimension gels, they were immediately run at 50 mA (constant)-500 V-85 W for 20 min, followed by 200 mA (constant)-500 V-85 W until the buffer front line was 10 to 15 mm from the bottom of the gel. Gels were removed and silver stained according to the procedure of Shevchenko et al. (29).

Protein identification. Gels were exposed to X-ray film overnight, and then the silver staining and film were used to excise 156 spots of varying intensities, molecular weights, and isoelectric focusing points. In order to increase the detection limit by mass spectrometry, spots were cut out and pooled from up to four identical cold, silver-stained gels. In-gel tryptic digests of pooled spots were performed as described previously (29). Tryptic peptides were analyzed by microcapillary LC-MS with automated switching to MS/MS mode for peptide fragmentation. Spectra were searched against the composite OWL protein sequence database (version 30.2; 250,514 protein sequences) (24a) by using the computer program Sequest (8), which matches theoretical and acquired tandem mass spectra. A protein match was determined by comparing the number of peptides identified and their respective cross-correlation scores. All protein identifications were verified by comparison with theoretical molecular weights and isoelectric points.

mRNA quantitation. Velculescu and coworkers have previously generated frequency tables for yeast mRNA transcripts from the same strain grown under the same stated conditions as described herein (35). The SAGE technology is based on two main principles. First, a short sequence tag (15 bp) that contains sufficient information uniquely to identify a transcript is generated. A single tag is usually generated from each mRNA transcript in the cell which corresponds to 15 bp at the 3'-most cutting site for *Nla*III. Second, many transcript tags can be concatenated into a single molecule and then sequenced, revealing the identity of multiple tags simultaneously. Over 20,000 transcripts were sequenced from yeast strain YPH499 growing at mid-log phase on glucose. Assuming the previously derived estimate of 15,000 mRNA molecules per cell (16), this would represent a 1.3-fold coverage even for mRNA molecules present at a single copy per cell and would provide a 72% probability of detecting such transcripts. Computer software which took for input the gene detected, examined the nucleotide sequence, and performed the calculation as described by Velculescu and coworkers (35) was written. In practice, we found that for 21 of 128 (16%) genes examined viable mRNA levels from SAGE data could not be calculated. This was because (i) no CATG site was found in the open reading frame (ORF), (ii) a CATG site was found but the corresponding 10-bp putative SAGE tag was not found in the frequency tables, or (iii) identical putative SAGE tags were present for multiple genes (e.g., *TDH2_YEAST* and *TDH3_YEAST*).

TABLE 1. Expressed genes identified from 2D gel in Fig. 2

Mol wt	pI	Spot no.	YPD gene name ^a	Protein abundance (10 ³ copies/cell)	mRNA abundance (copies/cell)	Codon bias
17,259	6.75	133	CPR1	15.2	61.7	0.769
18,702	4.80	83	EGD2	20.1	5.2	0.724
18,726	4.44	147	YKL056C	61.2	88.4	0.831
18,978	5.95	135	YER067W	3.7	6.7	0.118
19,108	5.04	130	YLR109W	94.4	9.7	0.680
19,681	9.08	136	ATP7	11.0	NA ^{b,c}	0.246
20,505	6.07	111	GUK1	16.5	3.7	0.422
21,444	5.25	148	SAR1	5.4	10.4	0.455
21,583	4.98	95	TSA1	110.6	40.1	0.845
22,602	4.30	80	EFB1	66.1	23.8	0.875
23,079	6.29	112	SOD2	12.6	2.2	0.351
23,743	5.44	137	HSP26	NA ^d	0.7	0.434
24,033	5.97	96	ADK1	17.4	16.4	0.656
24,058	4.43	143	YKL117W	29.2	10.4	0.339
24,353	6.30	140	TFS1	8.1	0.7	0.146
24,662	5.85	99	URA5	25.4	6.0	0.359
24,808	6.33	97	GSP1	26.3	5.2	0.735
24,908	8.73	122	RPS5	18.6	NA ^c	0.899
25,081	4.65	81	MRP8	9.3	NA ^c	0.241
25,960	6.06	116	RPE1	5.8	0.7	0.372
26,378	9.55	127	RPS3	96.8	NA ^c	0.863
26,467	5.18	100	VMA4	10.5	3.7	0.427
26,661	5.84	98	TPI1	NA ^d	NA ^c	0.900
27,156	5.56	93	PRE8	6.9	0.7	0.129
27,334	6.13	115	YHR049W	18.4	2.2	0.520
27,472	5.33	92	YNL010W	31.6	3.7	0.421
27,480	8.95	123	GPM1	10.0	169.4	0.902
27,480	8.95	124	GPM1	231.4	169.4	0.902
27,480	8.95	125	GPM1	7.5	169.4	0.902
27,809	5.97	139	HOR2	5.7	0.7	0.381
27,874	4.46	78	YST1	13.6	52.8	0.805
28,595	4.51	41	PUP2	4.4	0.7	0.147
29,156	6.59	114	YMR226C	14.5	2.2	0.283
29,244	8.40	120	DPM1	5.0	11.2	0.362
29,443	5.91	48	PRE4	3.4	3.7	0.162
30,012	6.39	138	PRB1	21.2	1.5	0.449
30,073	4.63	77	BMH1	14.7	28.2	0.454
30,296	7.94	121	OMP2	67.4	41.6	0.499
30,435	6.34	89	GPP1	70.2	11.2	0.703
31,332	5.57	88	ILV6	13.9	3.0	0.402
32,159	5.46	113	IPP1	63.1	3.7	0.752
32,263	6.00	149	HIS1	22.4	4.5	0.232
33,311	5.35	84	SPE3	15.1	6.7	0.468
34,465	5.60	129	ADE1	8.7	5.2	0.305
34,762	5.32	85	SEC14	10.9	6.0	0.373
34,797	5.85	42	URA1	49.5	8.9	0.237
34,799	6.04	90	BEL1	103.2	81.0	0.875
35,556	5.97	43	YDL124W	6.4	4.5	0.206
35,619	8.41	59	TDH1	69.8	32.7 ^c	0.940
35,650	5.49	68	CAR1	5.2	3.0	0.339
35,712	6.72	117	TDH2	49.6	473.0 ^c	0.982
35,712	6.72	154	TDH2	863.5	473.0 ^c	0.982
35,712	6.72	155	TDH2	79.4	473.0 ^c	0.982
36,272	4.85	128	APA1	8.7	0.7	0.425
36,358	5.05	75	YJR105W	17.6	17.1	0.522
36,358	5.05	76	YJR105W	27.5	17.1	0.522
36,596	6.37	79	ADH2	58.9	260.0 ^c	0.711
36,714	6.30	102	ADH1	746.1	260.0	0.913
36,714	6.30	103	ADH1	17.6	260.0	0.913
36,714	6.30	104	ADH1	61.4	260.0	0.913
36,714	6.30	105	ADH1	52.7	260.0	0.913
37,033	6.23	44	TAL1	44.8	3.7	0.701
37,796	7.36	57	IDH2	29.4	6.7	0.330
37,886	6.49	106	ILV5	76.0	4.5	0.892
38,700	7.83	55	BAT1	30.9	11.2	0.469
38,702	6.24	46	QCR2	NA ^d	2.2	0.326

Continued

TABLE 1—Continued

Mol wt	pI	Spot no.	YPD gene name ^a	Protein abundance (10 ³ copies/cell)	mRNA abundance (copies/cell)	Codon bias
39,477	5.58	86	FBA1	17.8	183.6	0.935
39,477	5.58	87	FBA1	427.2	183.6	0.935
39,540	6.50	150	HOM2	60.3	4.5	0.592
39,561	6.12	156	PSA1	96.4	27.5	0.718
41,158	6.01	49	YNL134C	14.9	1.5	0.316
41,623	7.18	58	BAT2	19.0	8.9	0.250
41,728	7.29	110	ERG10	24.1	4.5	0.543
41,900	5.42	74	TOM40	22.3	2.2	0.375
42,402	6.29	45	CYS3	6.7	8.9	0.621
42,883	5.63	67	DYS1	15.8	5.2	0.526
43,409	6.31	107	SER1	10.5	1.5	0.292
43,421	5.59	91	ERG6	2.2	14.1	0.408
44,174	7.32	56	YBR025C	13.1	6.0	0.684
44,682	4.99	72	TIF1	2.9	39.4	0.834
44,707	7.77	108	PGK1	23.7	165.7	0.897
44,707	7.77	109	PGK1	315.2	165.7	0.897
46,080	6.72	30	CAR2	15.4	NA ^c	0.495
46,383	8.52	53	IDP1	7.7	0.7	0.436
46,553	5.98	47	IDP2	32.4	NA ^c	0.197
46,679	6.39	50	ENO1	35.4	0.7	0.930
46,679	6.39	51	ENO1	6.6	0.7	0.930
46,679	6.39	52	ENO1	2.2	0.7	0.930
46,773	5.82	63	ENO2	15.5	289.1	0.960
46,773	5.82	64	ENO2	635.5	289.1	0.960
46,773	5.82	65	ENO2	93.0	289.1	0.960
46,773	5.82	66	ENO2	31.0	289.1	0.960
47,402	6.09	126	COR1	2.5	0.7	0.422
47,666	8.98	54	AAT2	11.7	6.0	0.338
48,364	5.25	73	WTM1	74.5	13.4	0.365
48,530	6.20	61	MET17	38.1	29.0	0.576
48,904	5.18	69	LYS9	16.2	3.7	0.463
48,987	4.90	153	SUP45	29.6	11.9	0.377
49,727	5.47	70	PRO2	13.6	5.2	0.297
49,912	9.27	62	TEF2	558.5	282.0	0.932
50,444	5.67	35	YDR190C	4.8	2.2	0.228
50,837	6.11	32	YEL047C	3.8	1.5	0.387
50,891	4.59	151	TUB2	11.2	7.4	0.404
51,547	6.80	27	LPD1	18.9	2.2	0.351
52,216	7.25	29	SHM2	19.7	7.4	0.722
52,859	5.54	37	YFR044C	30.2	6.7	0.442
53,798	5.19	71	HXK2	26.5	7.4	0.756
53,803	6.05	145	GYP6	4.4	0.7	0.147
54,403	5.29	39	ALD6	37.7	2.2	0.664
54,403	5.29	40	ALD6	6.6	2.2	0.664
54,502	6.20	31	ADE13	6.3	1.5	0.417
54,543	7.75	25	PYK1	225.3	101.8	0.965
54,543	7.75	26	PYK1	39.8	101.8	0.965
55,221	6.66	146	YEL071W	16.3	3.0	0.244
55,295	4.35	134	PDI1	66.2	14.1	0.589
55,364	5.98	24	GLK1	22.6	6.0	0.237
55,481	7.97	118	ATP1	21.6	2.2	0.637
55,886	6.47	28	CYS4	22.2	NA ^c	0.444
56,167	5.83	33	ARO8	14.3	3.0	0.324
56,167	5.83	34	ARO8	9.1	3.0	0.324
56,584	6.36	20	CYB2	18.9	NA ^c	0.259
57,366	5.53	60	FRS2	2.3	0.7	0.451
57,383	5.98	144	ZWF1	5.6	0.7	0.215
57,464	5.49	36	THR4	21.4	3.7	0.508
57,512	5.50	7	SRV2	6.5	NA ^c	0.260
57,727	4.92	152	VMA2	33.7	8.9	0.546
58,573	6.47	17	ACH1	4.4	1.5	0.327
58,573	6.47	18	ACH1	5.4	1.5	0.327
61,353	5.87	21	PDC1	6.5	200.7	0.962
61,353	5.87	22	PDC1	303.2	200.7	0.962
61,353	5.87	23	PDC1	16.3	200.7	0.962
61,649	5.54	38	CCT8	2.2	1.5	0.271

Continued on following page

TABLE 1—Continued

Mol wt	pI	Spot no.	YPD gene name ^a	Protein abundance (10 ³ copies/cell)	mRNA abundance (copies/cell)	Codon bias
61,902	6.21	101	PDC5	4.3	NA ^c	0.828
62,266	6.19	16	ICL1	20.1	NA ^c	0.327
62,862	8.02	19	ILV3	5.3	4.5	0.548
63,082	6.40	119	PGM2	2.2	3.0	0.402
64,335	5.77	5	PAB1	30.4	1.5	0.616
66,120	5.42	8	STI1	6.7	0.7	0.313
66,120	5.42	9	STI1	6.4	0.7	0.313
66,450	5.29	141	SSB2	7.0	NA ^c	0.880
66,450	5.29	142	SSB2	2.3	NA ^c	0.880
66,456	5.23	10	SSB1	64.5	79.5	0.907
66,456	5.23	11	SSB1	59.0	79.5	0.907
66,456	5.23	12	SSB1	13.7	79.5	0.907
68,397	5.82	82	LEU4	3.1	3.0	0.407
69,313	4.90	13	SSA2	24.3	18.6	0.892
69,313	4.90	14	SSA2	77.1	18.6	0.892
74,378	8.46	15	YKL029C	2.8	3.7	0.353
75,396	5.82	6	GRS1	5.5	7.4	0.500
85,720	6.25	1	MET6	2.0	NA ^c	0.772
85,720	6.25	2	MET6	10.9	NA ^c	0.772
85,720	6.25	3	MET6	1.4	NA ^c	0.772
93,276	6.11	131	EFT1	17.9	41.6	0.890
93,276	6.11	132	EFT1	5.7	41.6	0.890
102,064 ^e	6.61 ^e	94	ADE3	4.8	5.2	0.423
107,482 ^e	5.33 ^e	4	MCM3	2.7	NA ^c	0.240

^a YPD gene names are available from the YPD website (39).

^b NA, calculation could not be performed or was not available.

^c mRNA data inconclusive or NA.

^d No methionines in predicted ORF; therefore, protein concentration was not determined.

^e Measured molecular weight or pI did not match theoretical molecular weight or pI.

Protein quantitation. [³⁵S]methionine-labeled gels were exposed to X-ray film overnight, and then the silver stain and film were used to excise 156 spots of varying intensities, molecular weights, and pIs. The excised spots were placed in 0.6-ml microcentrifuge tubes, and scintillation cocktail (100 μ l) was added. The samples were vortexed and counted. In addition, two parallel gels were electroblotted to polyvinylidene difluoride membranes. The membranes were exposed to X-ray film, and four intense single spots were excised from each membrane and subjected to amino acid analysis. For these four spots, a mean of 209 ± 4 cpm/pmol of protein/methionine was found. This number was used to quantitate all remaining spots in conjunction with the number of methionines present in the protein.

To ensure that proteins were labeled to equilibrium, parallel 2D gels were prepared and run on yeast metabolically labeled for 1, 2, 6, or 18 h. The corresponding 156 spots were excised from each gel, and radioactivity was measured by liquid scintillation counting for each spot. Calculated protein levels were highly reproducible for all time points measured after 1 h.

Calculation of codon bias and predicted half-life. Codon bias values were extracted from the YPD spreadsheet (17). Protein half-lives were calculated based on the N-end rule (33). When the N-terminal processing was not known experimentally, it was predicted based on the affinity of methionine aminopeptidase (31).

RESULTS

Characteristics of proteome approach. Nearly every facet of proteome analysis hinges on the unambiguous identification of large numbers of expressed proteins in cells. Several techniques have been described previously for the identification of proteins separated by 2DE, including N-terminal and internal sequencing (1, 2), amino acid analysis (38), and more recently mass spectrometry (25). We utilized techniques based on mass spectrometry because they afford the highest levels of sensitivity and provide unambiguous identification. The specific procedure used is schematically illustrated in Fig. 1 and is based on three principles. First, proteins are removed from the gel by

proteolytic in-gel digestion, and the resulting peptides are separated by on-line capillary high-performance liquid chromatography. Second, the eluting peptides are ionized and detected, and the specific peptide ions are selected and fragmented by the mass spectrometer. To achieve this, the mass spectrometer switches between the MS mode (for peptide mass identification) and the MS/MS mode (for peptide characterization and sequencing). Selected peptides are fragmented by a process called collision-induced dissociation (CID) to generate a tandem mass spectrum (MS/MS spectrum) that contains the peptide sequence information. Third, individual CID mass spectra are then compared by computer algorithms to predicted spectra from a sequence database. This results in the identification of the peptide and, by association, the protein(s) in the spot. Unambiguous protein identification is attained in a single analysis by the detection of multiple peptides derived from the same protein.

Protein identification. Yeast total cell protein lysate (40 μ g), metabolically labeled with [³⁵S]methionine, was electrophoretically separated by isoelectric focusing in the first dimension and by SDS-10% polyacrylamide gel electrophoresis in the second dimension. Proteins were visualized by silver staining and by autoradiography. Of the more than 1,000 proteins visible by silver staining, 156 spots were excised from the gel and subjected to in-gel tryptic digestion, and the resulting peptides were analyzed and identified by microspray LC-MS/MS techniques as described above. The proteins in this study were all identified automatically by computer software with no human interpretation of mass spectra. They are indicated in Fig. 2 and detailed in Table 1.

The CID spectra shown in Fig. 3 indicate that the quality of the identification data generated was suitable for unambiguous protein identification. The spectra represent the amino acid sequences of tryptic peptides NSGDIVNLGSIAGR (Fig. 3A) and FAVGAFTDSLRL (Fig. 3B). Both peptides were derived from protein S57593 (hypothetical protein YMR226C), which migrated to spot 114 (molecular weight, 29,156; pI, 6.59) in the 2D gel in Fig. 2. Five other peptides from the same analysis were also computer matched to the same protein sequence.

Protein and mRNA quantitation. For the 156 genes investigated, the protein expression levels ranged from 2,200 (PGM2) to 863,000 (TDH2/TDH3) copies/cell. The levels of mRNA for each of the genes identified were calculated from SAGE frequency tables (35). These tables contain the mRNA levels for 4,665 genes in yeast strain YPH499 grown to mid-log phase in YPD medium on glucose as a carbon source. In some instances, the mRNA levels could not be calculated for reasons stated in Materials and Methods. For the proteins analyzed in this study, mean transcript levels varied from 0.7 to 473 copies/cell.

Selection of the sample population for mRNA-protein expression level correlation. The protein spots selected for identification were selected from spots visible by silver staining in the 2D gel. An attempt was made not to include spots where overlap with other spots was readily apparent. The number of proteins identified was 156 (Table 1). Some proteins migrated to more than one spot (presumably due to differential protein processing or modifications), and protein levels from these spots were calculated by integrating the intensities of the different spots. The 156 protein spots analyzed represented the products of 128 different genes. Genes were excluded from the correlation analysis only if part of the data set was missing; i.e., genes were excluded if (i) no mRNA expression data were available for the protein or putative SAGE tags were ambiguous, (ii) the amino acid sequence did not contain methionine, (iii) more than a single protein was conclusively identified as

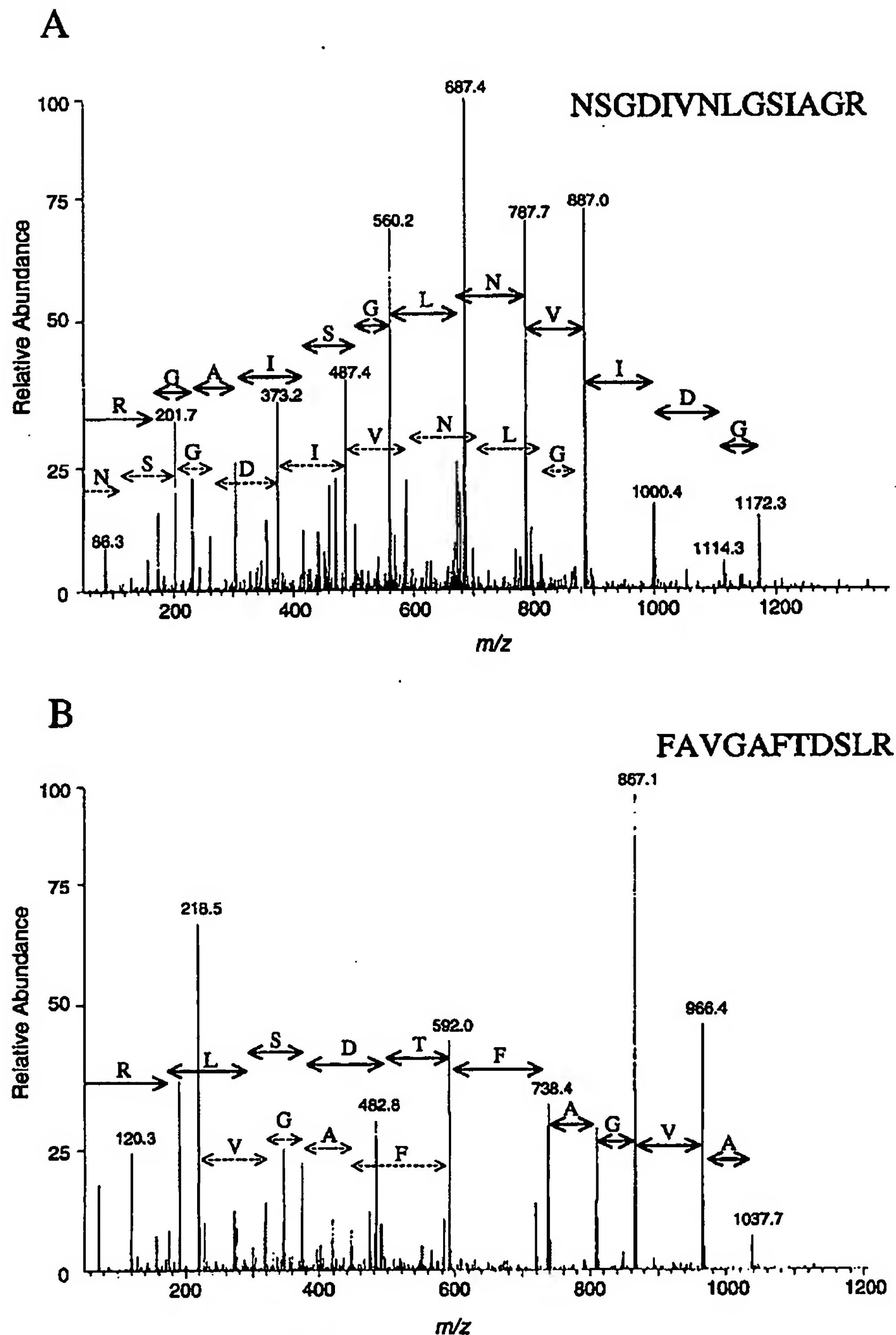


FIG. 3. Tandem mass (MS/MS) spectra resulting from analysis of a single spot on a 2D gel. The first quadrupole selected a single mass-to-charge ratio (m/z) of 687.2 (A) or 592.6 (B), while the collision cell was filled with argon gas, and a voltage which caused the peptide to undergo fragmentation by CID was applied. The third quadrupole scanned the mass range from 50 to 1,400 m/z . The computer program Sequest (8) was utilized to match MS/MS spectra to amino acid sequence by database searching. Both spectra matched peptides from the same protein, S57593 (yeast hypothetical protein YMR226C). Five other peptides from the same analysis were matched to the same protein.

migrating to the same gel spot, or (iv) the theoretical and observed pIs and molecular weights could not be reconciled. After these criteria were applied, the number of genes used in the correlation analysis was 106.

Codon bias and predicted half-lives. Codon bias is thought to be an indicator of protein expression, with highly expressed proteins having large codon bias values. The codon bias distribution for the entire set of more than 6,000 predicted yeast

gene ORFs is presented in Fig. 4A. The interval with the largest frequency of genes is between the codon bias values of 0.0 and 0.1. This segment contains more than 2,500 genes. The distribution of the codon bias values of the 128 different genes found in this study (all protein spots from Fig. 2) is shown in Fig. 4B, and protein half-lives (predicted from applying the N-end rule [33] to the experimentally determined or predicted protein N termini) are shown in Fig. 4C. No genes were identified with codon bias values less than 0.1 even though thousands of genes exist in this category. In addition, nearly all of the proteins identified had long predicted half-lives (greater than 30 h).

Correlation of mRNA and protein expression levels. The correlation between mRNA and protein levels of the genes selected as described above is shown in Fig. 5. For the entire group (106 genes) for which a complete data set was generated, there was a general trend of increased protein levels resulting from increased mRNA levels. The Pearson product moment correlation coefficient for the whole data set (106 genes) was 0.935. This number is highly biased by a small number of genes with very large protein and message levels. A more representative subset of the data is shown in the inset of Fig. 5. It shows genes for which the message level was below 10 copies/cell and includes 69% (73 of 106 genes) of the data used in the study. The Pearson product moment correlation coefficient for this data set was only 0.356. We also found that levels of protein expression coded for by mRNA with comparable abundance varied by as much as 30-fold and that the mRNA levels coding for proteins with comparable expression levels varied by as much as 20-fold.

The distortion of the correlation value induced by the uneven distribution of the data points along the x axis is further demonstrated by the analysis in Fig. 6. The 106 samples included in the study were ranked by protein abundance, and the Pearson product moment correlation coefficient was repeatedly calculated after including progressively more, and higher-abundance, proteins in each calculation. The correlation values remained relatively stable in the range of 0.1 to 0.4 if the lowest-expressed 40 to 95 proteins used in this study were included. However, the correlation value steadily climbed by the inclusion of each of the 11 very highly expressed proteins.

Correlation of protein and mRNA expression levels with codon bias. Codon bias is the propensity for a gene to utilize the same codon to encode an amino acid even though other codons would insert the identical amino acid in the growing polypeptide sequence. It is further thought that highly expressed proteins have large codon biases (3). To assess the value of codon bias for predicting mRNA and protein levels in exponentially growing yeast cells, we plotted the two experimental sets of data versus the codon bias (Fig. 7). The distribution patterns for both mRNA and protein levels with respect to codon bias were highly similar. There was high variability in the data within the codon bias range of 0.8 to 1.0. Although a large codon bias generally resulted in higher protein and message expression levels, codon bias did not appear to be predictive of either protein levels or mRNA levels in the cell.

DISCUSSION

The desired end point for the description of a biological system is not the analysis of mRNA transcript levels alone but also the accurate measurement of protein expression levels and their respective activities. Quantitative analysis of global mRNA levels currently is a preferred method for the analysis of the state of cells and tissues (11). Several methods which either provide absolute mRNA abundance (34, 35) or relative

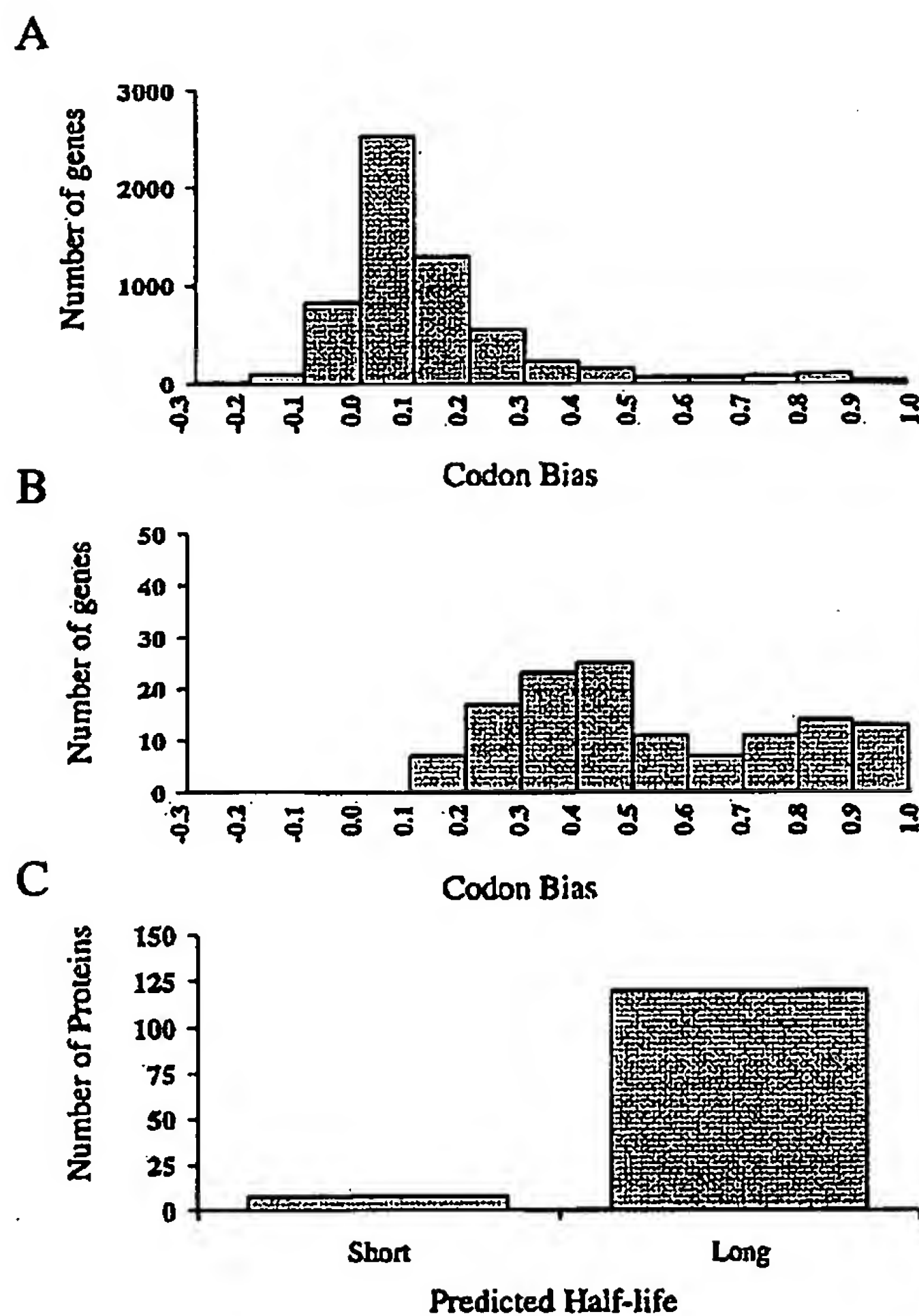


FIG. 4. Current proteome analysis technology utilizing 2DE without pre-enrichment samples mainly highly expressed and long-lived proteins. Genes encoding highly expressed proteins generally have large codon bias values. (A) Distribution of the yeast genome (more than 6,000 genes) based on codon bias. The interval with the largest frequency of genes is 0.0 to 0.1, with more than 2,500 genes. (B) Distribution of the genes from identified proteins in this study based on codon bias. No genes with codon bias values less than 0.1 were detected in this study. (C) Distribution of identified proteins in this study based on predicted half-life (estimated by N-end rule).

mRNA levels in comparative analyses (20, 27) have been described elsewhere. The techniques are fast and exquisitely sensitive and can provide mRNA abundance for potentially any expressed gene. Measured mRNA levels are often implicitly or explicitly extrapolated to indicate the levels of activity of the corresponding protein in the cell. Quantitative analysis of protein expression levels (proteome analysis) is much more time-consuming because proteins are analyzed sequentially one by one and is not general because analyses are limited to the relatively highly expressed proteins. Proteome analysis does, however, provide types of data that are of critical importance for the description of the state of a biological system and that are not readily apparent from the sequence and the level of expression of the mRNA transcript. This study attempts to examine the relationship between mRNA and protein expression levels for a large number of expressed genes in cells representing the same state.

Limits in the sensitivity of current protein analysis technology precluded a completely random sampling of yeast proteins. We therefore based the study on those proteins visible by silver

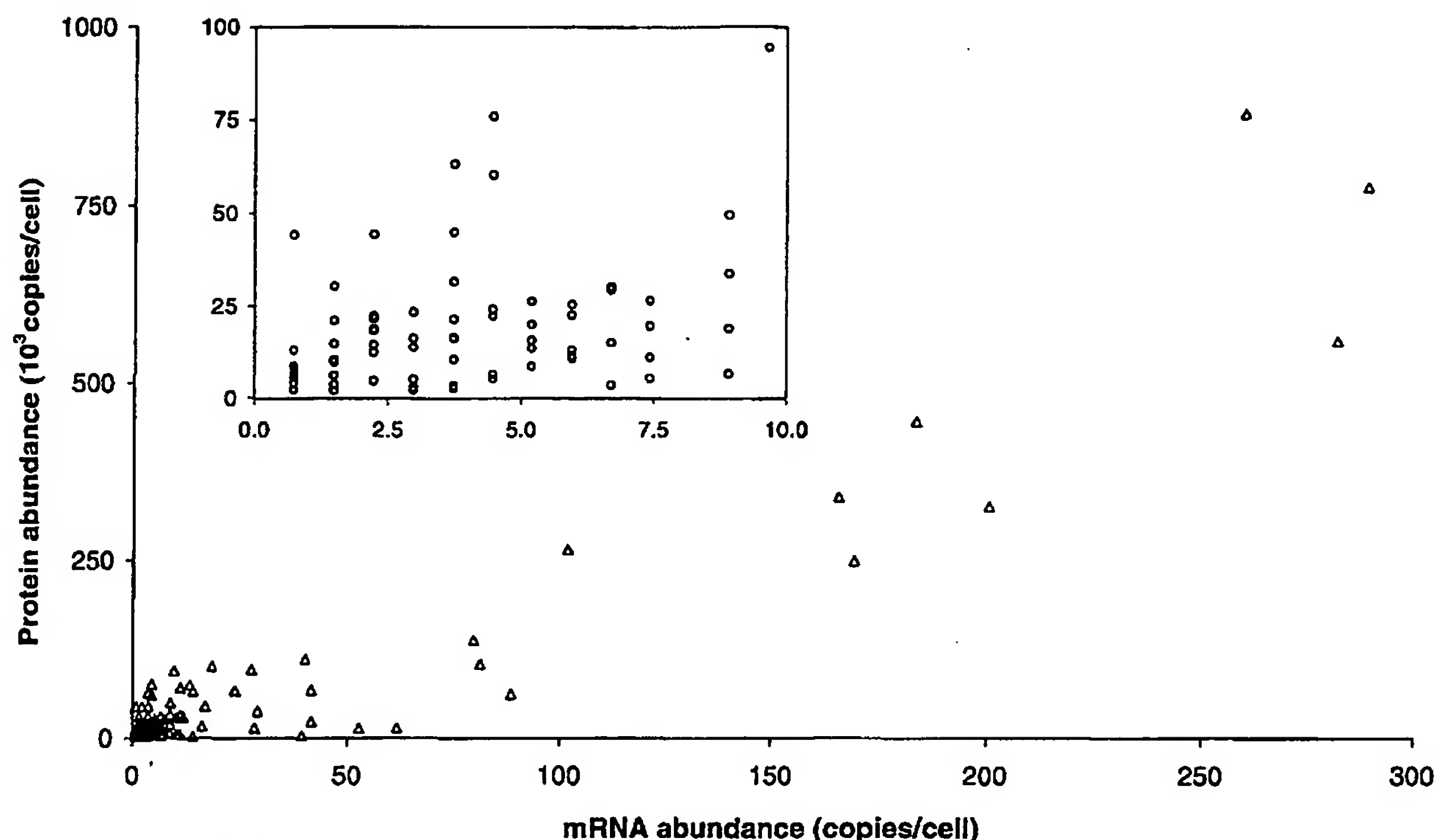


FIG. 5. Correlation between protein and mRNA levels for 106 genes in yeast growing at log phase with glucose as a carbon source. mRNA and protein levels were calculated as described in Materials and Methods. The data represent a population of genes with protein expression levels visible by silver staining on a 2D gel chosen to include the entire range of molecular weights, isoelectric focusing points, and staining intensities. The inset shows the low-end portion of the main figure. It contains 69% of the original data set. The Pearson product moment correlation for the entire data set was 0.935. The correlation for the inset containing 73 proteins (69%) was only 0.356.

staining on a 2D gel. Of the more than 1,000 visible spots, 156 were chosen to include the entire range of molecular weights, isoelectric focusing points, and staining intensities displayed on the 2D protein pattern. The genes identified in this study shared a number of properties. First, all of the proteins in this study had a codon bias of greater than 0.1 and 93% were greater than 0.2 (Fig. 4B). Second, with few exceptions, the proteins in this study had long predicted half-lives according to the N-end rule (Fig. 4C). Third, low-abundance proteins with regulatory functions such as transcription factors or protein kinases were not identified.

Because the population of proteins used in this study appears to be fairly homogeneous with respect to predicted half-life and codon bias, it might be expected that the correlation of the mRNA and protein expression levels would be stronger for this population than for a random sample of yeast proteins. We tested this assumption by evaluating the correlation value if different subsets of the available data were included in the calculation. The 106 proteins were ranked from lowest to highest protein expression level, and the trend in the correlation value was evaluated by progressively including more of the higher-abundance proteins in the calculation (Fig. 6). The correlation value when only the lower-abundance 40 to 93 proteins were examined was consistently between 0.1 and 0.4. If the 11 most abundant proteins were included, the correlation steadily increased to 0.94. We therefore expect that the correlation for all yeast proteins or for a random selection would be less than 0.4. The observed level of correlation between mRNA and protein expression levels suggests the importance

of posttranslational mechanisms controlling gene expression. Such mechanisms include translational control (15) and control of protein half-life (33). Since these mechanisms are also active in higher eukaryotic cells, we speculate that there is no predictive correlation between steady-state levels of mRNA and those of protein in mammalian cells.

Like other large-scale analyses, the present study has several potential sources of error related to the methods used to determine mRNA and protein expression levels. The mRNA levels were calculated from frequency tables of SAGE data. This method is highly quantitative because it is based on actual sequencing of unique tags from each gene, and the number of times that a tag is represented is proportional to the number of mRNA molecules for a specific gene. This method has some limitations including the following: (i) the magnitude of the error in the measurement of mRNA levels is inversely proportional to the mRNA levels, (ii) SAGE tags from highly similar genes may not be distinguished and therefore are summed, (iii) some SAGE tags are from sequences in the 3' untranslated region of the transcript, (iv) incomplete cleavage at the SAGE tag site by the restriction enzyme can result in two tags representing one mRNA, and (v) some transcripts actually do not generate a SAGE tag (34, 35).

For the SAGE method, the error associated with a value increases with a decreasing number of transcripts per cell. The conclusions drawn from this study are dependent on the quality of the mRNA levels from previously published data (35). Since more than 65% of the mRNA levels included in this study were calculated to 10 copies/cell or less (40% were less

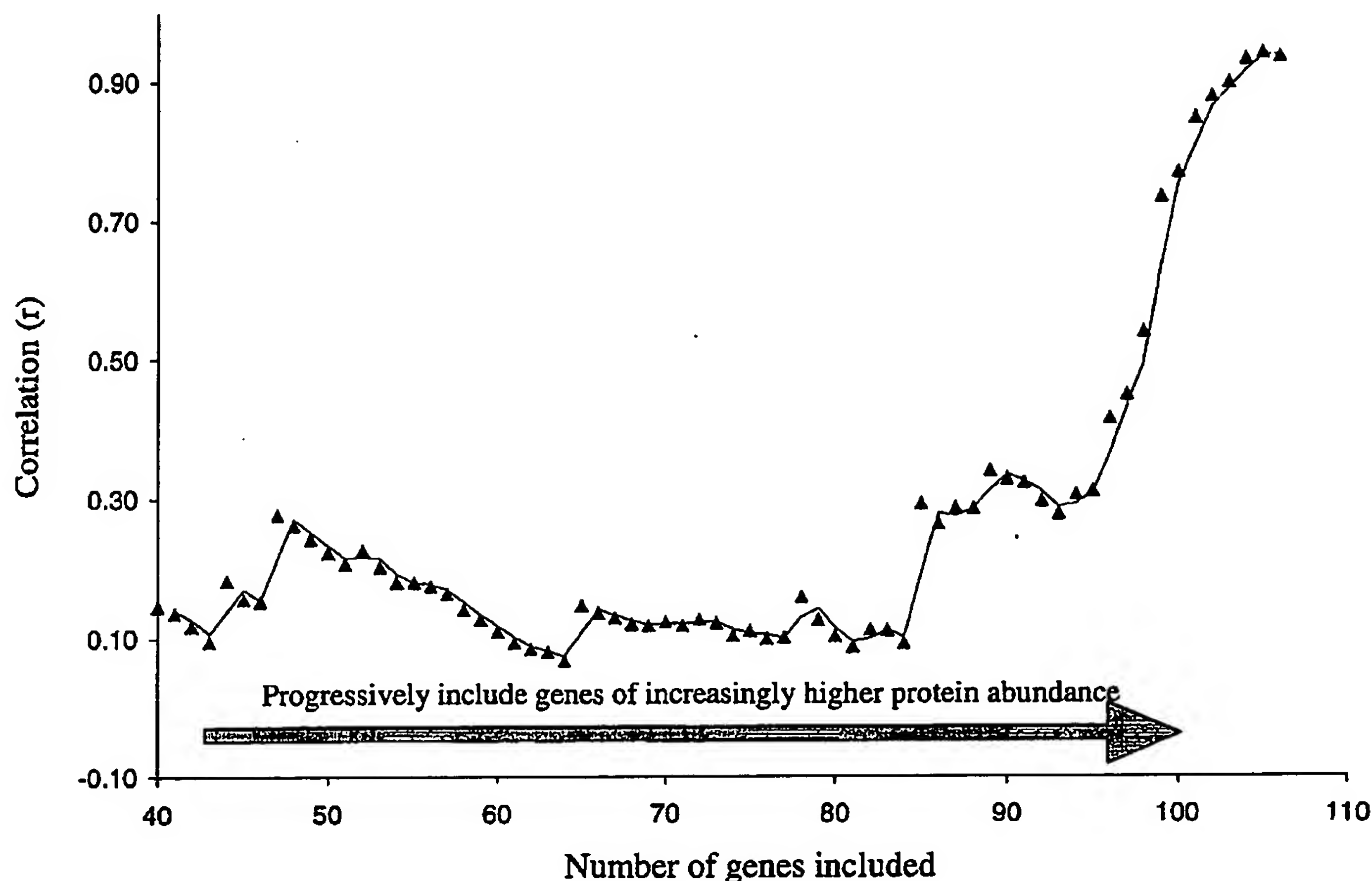


FIG. 6. Effect of highly abundant proteins on Pearson product moment correlation coefficient for mRNA and protein abundance in yeast. The set of 106 genes was ranked according to protein abundance, and the correlation value was calculated by including the 40 lowest-abundance genes and then progressively including the remaining 66 genes in order of abundance. The correlation value climbs as the final 11 highly abundant proteins are included.

than 4 copies/cell), the error associated with these values may be quite large. The mRNA levels were calculated from more than 20,000 transcripts. Assuming that the estimate of 15,000 mRNA molecules per cell is correct (16), this would mean that mRNA transcripts present at only a single copy per cell would be detected 72% of the time (35). The mRNA levels for each gene were carefully scrutinized, and only mRNA levels for which a high degree of confidence existed were included in the correlation value.

Protein abundance was determined by metabolic radiolabeling with [^{35}S]methionine. The calculation required knowledge of three variables: the number of methionines in the mature protein, the radioactivity contained in the protein, and the specific activity of the radiolabel normalized per methionine. The number of methionines per protein was determined from the amino acid sequence of the proteins identified by tandem mass spectrometry. For some proteins, it was not known whether the methionine of the nascent polypeptide was processed away. The N termini of those proteins were predicted based on the specificity of methionine aminopeptidase (31). If the N-terminal processing did not conform to the predicted specificity of processing enzymes, the calculation of the number of methionines would be affected. This discrepancy would affect most the quantitation of a protein with a very low number of methionines. The average number of calculated methionines per protein in this study was 7.2. We therefore expect the potential for erroneous protein quantitation due to unusual N-terminal processing to be small.

The amount of radioactivity contained in a single spot might be the sum of the radioactivity of comigrating proteins. Because protein identification was based on tandem mass spectrometric techniques, comigrating proteins could be identified. However, comigrating proteins were rarely detected in this study, most likely because relatively small amounts of total protein (40 μg) were initially loaded onto the gels, which resulted in highly focused spots containing generally 1 to 25 ng of protein. Because of the relatively small amount loaded, the concentrations of any potentially comigrating protein would likely be below the limit of detection of the mass spectrometry technique used in this study (1 to 5 ng) and below the limit of visualization by silver staining (1 to 5 ng). In the overwhelming majority of the samples analyzed, numerous peptides from a single protein were detected. It is assumed that any comigrating proteins were at levels too low to be detected and that their influence in the calculation would be small.

The specific activity of the radiolabel was determined by relating the precise amount of protein present in selected spots of a parallel gel, as determined by quantitative amino acid composition analysis, to the number of methionines present in the sequence of those proteins and the radioactivity determined by liquid scintillation counting. It is possible that the resulting number might be influenced by unavoidable losses inherent in the amino acid analysis procedure applied. Because four different proteins were utilized in the calculation and the experiment was done in duplicate, the specific activity calculated is thought to be highly accurate. Indeed, the specific

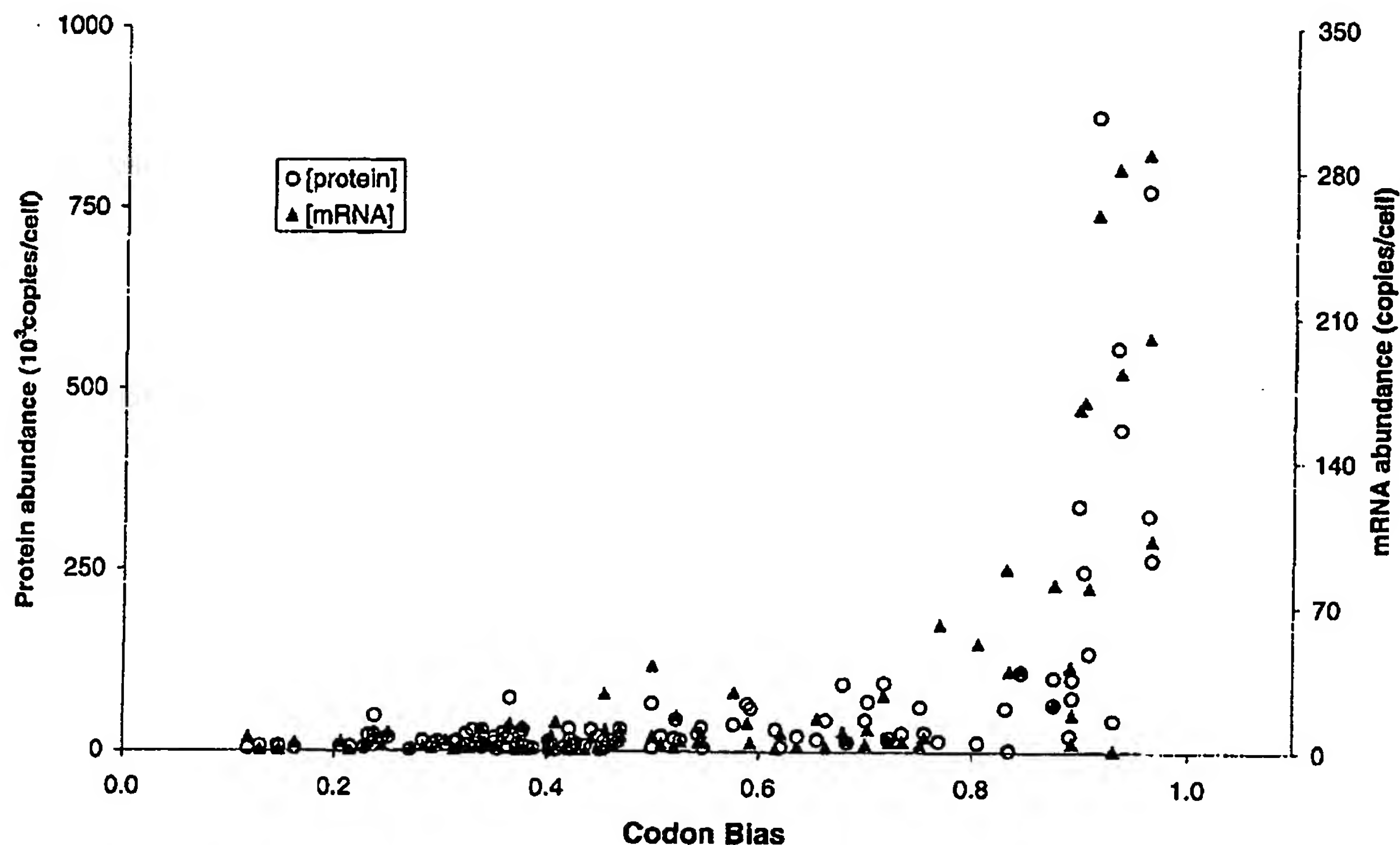


FIG. 7. Relationship between codon bias and protein and mRNA levels in this study. Yeast mRNA and protein expression levels were calculated as described in Materials and Methods. The data represent the same 106 genes as in Fig. 5.

activities calculated for each of the four proteins varied by less than 10%. Any inconsistencies in the calculation of the specific activity would result in differences in the absolute levels calculated but not in the relative numbers and would therefore not influence the correlation value determined.

The protein quantitative method used eliminates a number of potential errors inherent in previous methods for the quantitation of proteins separated by 2DE, such as preferential protein staining and bias caused by inequalities in the number of radiolabeled residues per protein. Any 2D gel-based method of quantitation is complicated by the fact that in some cases the translation products of the same mRNA migrated to different spots. One major reason is posttranslational modification or processing of the protein. Also, artifactual proteolysis during cell lysis and sample preparation can lead to multiple resolved forms of the protein. In such cases, the protein levels of spots coded for by the same mRNA were pooled. In addition, the existence of other spots coded for by the same mRNA that were not analyzed by mass spectrometry or that were below the limit of detection for silver staining cannot be ruled out. However, since this study is based on a class of highly expressed proteins, the presence of undetected minor spots below silver staining sensitivity corresponding to a protein analyzed in the study would generally cause a relatively small error in protein quantitation.

Codon bias is a measure of the propensity of an organism to selectively utilize certain codons which result in the incorporation of the same amino acid residue in a growing polypeptide chain. There are 61 possible codons that code for 20 amino acids. The larger the codon bias value, the smaller the number of codons that are used to encode the protein (19). It is

thought that codon bias is a measure of protein abundance because highly expressed proteins generally have large codon bias values (3, 13).

Nearly all of the most highly expressed proteins had codon bias values of greater than 0.8. However, we detected a number of genes with high codon bias and relative low protein abundance (Fig. 7). For example, the expressed gene with both the second largest protein and mRNA levels in the study was ENO2_YEAST (775,000 and 289.1 copies/cell, respectively). ENO1_YEAST was also present in the gel at much lower protein and mRNA levels (44,200 and 0.7 copies/cell, respectively). The codon bias values for ENO2 and ENO1 are similar (0.96 and 0.93, respectively), but the expression of the two genes is differentially regulated. Specifically, ENO1_YEAST is glucose repressed (6) and was therefore present in low abundance under the conditions used. Other genes with large codon bias values that were not of high protein abundance in the gel include EFT1, TIF1, HXK2, GSP1, EGD2, SHM2, and TAL1. We conclude that merely determining the codon bias of a gene is not sufficient to predict its protein expression level.

Interestingly, codon bias appears to be an excellent indicator of the boundaries of current 2D gel proteome analysis technology. There are thousands of genes with expressed mRNA and likely expressed protein with codon bias values less than 0.1 (Fig. 4A). In this study, we detected none of them, and only a very small percentage of the genes detected in this study had codon bias values between 0.1 and 0.2 (Fig. 4B). Indeed, in every examined yeast proteome study (5, 7, 13, 28) where the combined total number of identified proteins is 300 to 400, this same observation is true. It is expected that for the more complex cells of higher eukaryotic organisms the detection of

low-abundance proteins would be even more challenging than for yeast. This indicates that highly abundant, long-lived proteins are overwhelmingly detected in proteome studies. If proteome analysis is to provide truly meaningful information about cellular processes, it must be able to penetrate to the level of regulatory proteins, including transcription factors and protein kinases. A promising approach is the use of narrow-range focusing gels with immobilized pH gradients (IPG) (23). This would allow for the loading of significantly more protein per pH unit covered and also provide increased resolution of proteins with similar electrophoretic mobilities. A standard pH gradient in an isoelectric focusing gel covers a 7-pH-unit range (pH 3 to 10) over 18 cm. A narrow-range focusing gel might expand the range to 0.5 pH units over 18 cm or more. This could potentially increase by more than 10-fold the number of proteins that can be detected. Clearly, current proteome technology is incapable of analyzing low-abundance regulatory proteins without employing an enrichment method for relatively low-abundance proteins. In conclusion, this study examined the relationship between yeast protein and message levels and revealed that transcript levels provide little predictive value with respect to the extent of protein expression.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation Science and Technology Center for Molecular Biotechnology, NIH grant T32HG00035-3, and a grant from Oxford Glycosciences.

We thank Jimmy Eng for expert computer programming, Garry Corthals and John R. Yates III for critical discussion, and Siavash Mohandesi for expert technical help.

REFERENCES

- Aebersold, R. H., J. Leavitt, R. A. Saavedra, L. E. Hood, and S. B. Kent. 1987. Internal amino acid sequence analysis of proteins separated by one- or two-dimensional gel electrophoresis after in situ protease digestion on nitrocellulose. *Proc. Natl. Acad. Sci. USA* 84:6970-6974.
- Aebersold, R. H., D. B. Teplow, L. E. Hood, and S. B. Kent. 1986. Electrophoretic transfer onto activated glass. High efficiency preparation of proteins from analytical sodium dodecyl sulfate-polyacrylamide gels for direct sequence analysis. *Eur. J. Biochem.* 261:4229-4238.
- Bennetzen, J. L., and B. D. Hall. 1982. Codon selection in yeast. *J. Biol. Chem.* 257:3026-3031.
- Boucherle, H., G. Dujardin, M. Kermorgant, C. Monribot, P. Slonimski, and M. Perrot. 1995. Two-dimensional protein map of *Saccharomyces cerevisiae*: construction of a gene-protein index. *Yeast* 11:601-613.
- Boucherle, H., F. Sagliocco, R. Joubert, L. Maillet, J. Labarre, and M. Perrot. 1996. Two-dimensional gel protein database of *Saccharomyces cerevisiae*. *Electrophoresis* 17:1683-1699.
- Carmen, A. A., P. K. Bridle, C. S. Park, and M. J. Holland. 1995. Transcriptional regulation by an upstream repression sequence from the yeast enolase gene *ENO1*. *Yeast* 11:1031-1043.
- Ducet, A., L. VanOostveen, J. K. Eng, J. R. Yates, and R. Aebersold. 1998. High throughput protein characterization by automated reverse-phase chromatography/electrospray tandem mass spectrometry. *Protein Sci.* 7:706-719.
- Eng, J., A. McCormack, and J. R. Yates. 1994. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* 5:976-989.
- Figgeys, D., A. Ducet, J. R. Yates, and R. Aebersold. 1996. Protein identification by solid phase microextraction-capillary zone electrophoresis-microelectrospray-tandem mass spectrometry. *Nat. Biotechnol.* 14:1579-1583.
- Figgeys, D., I. VanOostveen, A. Ducet, and R. Aebersold. 1996. Protein identification by capillary zone electrophoresis/microelectrospray ionization-tandem mass spectrometry at the subfemtomole level. *Anal. Chem.* 68:1822-1828.
- Fraser, C. M., and R. D. Fleischmann. 1997. Strategies for whole microbial genome sequencing and analysis. *Electrophoresis* 18:1207-1216.
- Garrels, J. I., B. Futcher, R. Kobayashi, G. I. Latter, B. Schwender, T. Volpe, J. R. Warner, and C. S. McLaughlin. 1994. Protein identifications for a *Saccharomyces cerevisiae* protein database. *Electrophoresis* 15:1466-1486.
- Garrels, J. I., C. S. McLaughlin, J. R. Warner, B. Futcher, G. I. Latter, R. Kobayashi, B. Schwender, T. Volpe, D. S. Anderson, F. Mesquita-Fuentes, and W. E. Payne. 1997. Proteome studies of *Saccharomyces cerevisiae*: identification and characterization of abundant proteins. *Electrophoresis* 18:1347-1360.
- Gygi, S. P., and R. Aebersold. 1998. Absolute quantitation of 2-DE protein spots, p. 417-421. In A. J. Link (ed.), 2-D protocols for proteome analysis. Humana Press, Totowa, N.J.
- Harford, J. B., and D. R. Morris. 1997. Post-transcriptional gene regulation. Wiley-Liss, Inc., New York, N.Y.
- Hereford, L. M., and M. Rosbash. 1977. Number and distribution of polyadenylated RNA sequences in yeast. *Cell* 10:453-462.
- Hodges, P. E., W. E. Payne, and J. I. Garrels. 1998. The Yeast Protein Database (YPD): a curated proteome database for *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 26:68-72.
- Klose, J., and U. Kobalz. 1995. Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. *Electrophoresis* 16:1034-1059.
- Kurland, C. G. 1991. Codon bias and gene expression. *FEBS Lett.* 285:165-169.
- Lashkari, D. A., J. L. DeRisi, J. H. McCusker, A. F. Namath, C. Gentile, S. Y. Hwang, P. O. Brown, and R. W. Davis. 1997. Yeast microarrays for genome wide parallel genetic and gene expression analysis. *Proc. Natl. Acad. Sci. USA* 94:13057-13062.
- Liang, P., and A. B. Pardee. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967-971.
- Link, A. J., L. G. Hays, E. B. Carmack, and J. R. Yates III. 1997. Identifying the major proteome components of *Haemophilus influenzae* type-strain NCTC 8143. *Electrophoresis* 18:1314-1334.
- Nawrocki, A., M. R. Larsen, A. V. Podtelejnikov, O. N. Jensen, M. Mann, P. Roepstorff, A. Gorg, S. J. Fey, and P. M. Larsen. 1998. Correlation of acidic and basic carrier ampholyte and immobilized pH gradient two-dimensional gel electrophoresis patterns based on mass spectrometric protein identification. *Electrophoresis* 19:1024-1035.
- O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.
- OWL Protein Sequence Database. 2 August 1998, posting date. [Online.] <http://bmbg111.leeds.ac.uk/bmb5dp/owl.html>. [8 January 1999, last date accessed.]
- Patterson, S. D., and R. Aebersold. 1995. Mass spectrometric approaches for the identification of gel-separated proteins. *Electrophoresis* 16:1791-1814.
- Pennington, S. R., M. R. Wilkins, D. F. Hochstrasser, and M. J. Dunn. 1997. Proteome analysis: from protein characterization to biological function. *Trends Cell Biol.* 7:168-173.
- Shalon, D., S. J. Smith, and P. O. Brown. 1996. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. *Genome Res.* 6:639-645.
- Shevchenko, A., O. N. Jensen, A. V. Podtelejnikov, F. Sagliocco, M. Wilm, O. Vorm, P. Mortensen, H. Boucherle, and M. Mann. 1996. Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. *Proc. Natl. Acad. Sci. USA* 93:14440-14445.
- Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* 68:850-858.
- Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122:19-27.
- Tsunasawa, S., J. W. Stewart, and F. Sherman. 1985. Amino-terminal processing of mutant forms of yeast iso-1-cytochrome c. The specificities of methionine aminopeptidase and acetyltransferase. *J. Biol. Chem.* 260:5382-5391.
- Urlinger, S., K. Kuchler, T. H. Meyer, S. Uebel, and R. Tamp'e. 1997. Intracellular location, complex formation, and function of the transporter associated with antigen processing in yeast. *Eur. J. Biochem.* 245:266-272.
- Varshavsky, A. 1996. The N-end rule: functions, mysteries, uses. *Proc. Natl. Acad. Sci. USA* 93:12142-12149.
- Velculescu, V. E., L. Zhang, B. Vogelstein, and K. W. Kinzler. 1995. Serial analysis of gene expression. *Science* 270:484-487.
- Velculescu, V. E., L. Zhang, W. Zhou, J. Vogelstein, M. A. Basrai, D. E. Bassett, Jr., P. Hieter, B. Vogelstein, and K. W. Kinzler. 1997. Characterization of the yeast transcriptome. *Cell* 88:243-251.
- Wilkins, M. R., K. L. Williams, R. D. Appel, and D. F. Hochstrasser. 1997. Proteome research: new frontiers in functional genomics. Springer-Verlag, Berlin, Germany.
- Wilm, M., A. Shevchenko, T. Houthaeve, S. Breit, L. Schweigerer, T. Fotsis, and M. Mann. 1996. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* 379:466-469.
- Yan, J. X., M. R. Wilkins, K. Ou, A. A. Gooley, K. L. Williams, J. C. Sanchez, O. Golaz, C. Pasquali, and D. F. Hochstrasser. 1996. Large-scale amino-acid analysis for proteome studies. *J. Chromatogr. A* 736:291-302.
- YPD Website. 6 March 1998, revision date. [Online.] Proteome, Inc. <http://www.proteome.com/YPDhome.html>. [8 January 1999, last date accessed.]

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.